ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

CHEMOMETRICS ASSISTED UV- SPECTROPHOTOMETRIC DETERMINATION OF TOPICAL BINARY MIXTURES CONTAINING BENZOIC ACID, SALICYLIC ACID OR RESORCINOL

BY

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FEBRUARY, 2008

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A thesis Submitted to the School of Graduate Studies of Addis Ababa University in partial fulfilment of the requirement for the Degree of Master of Science in Pharmaceutical Analysis and Quality Assurance

February, 2008

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This thesis is dedicated to my mothers Tenagnework Adnew

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ACKNOWLEDGEMENTS

Above all, my priceless and prime veneration is attached the Almighty God for He granted me strength and health; and St. Virgin Mary for her intersession throughout my deeds.

I am highly grateful to express my sincere and deepest appreciation to the eminent and kind advisor Prof. Dr. Abdel-Maobound I Mohamd for providing me genuine professional comments and a great deal of effort throughout the entire study.

My gratitude also goes to AAU graduate studies for granting me a scholarship, and financial support for my thesis work. Moreover I would like to thank department of pharmaceutical chemistry for cordial cooperation.

I also wish to express my gratitude to EPHARM staff of QC, and Research department for laboratory provision .I am respectfully indebted to Sileshi Wongel department head of QA for his cordial and technical support.

Finally, I wish to extend my sincere thank to my sister Haregua Kassa who paid a due attention in writing, this thesis and providing me computer; and my brother Taglo Kassa for his computer provision.

Mulualem Kassa

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LIST OFABREVIATIONS

CLS	Classical least square
^{1}D	First derivative
DS	Derivative spectrophotometry
LOD	Limit of detection
LOQ	Limit of quantification
MLR	Multiple linear regression
PCA	Principal component analysis
PCR	Principal component regression
PLS	Partial least square
RSD	Relative standard deviation
SNR	Signal to noise ratio
UV	Ultra violet

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ABSTRACT

Chemometrics-assisted spectrophotometric methods were developed for simultaneous determinations of binary mixtures of (1). benzoic acid and salicylic acid, and (2). resorcinol and salicylic acid mixture. The commercial forms were marketed in Ethiopia and Egypt, such as whitefield ointment, luna soap, and dorin emulsion. The uv absorption spectra of the studied compounds in the range of 200-400 nm, showed considerable degrees of spectral overlapping (between 87.6% for benzoic acid and salicylic acid mixture, and 96.2% for salicylic acid and resorcinol mixture). The resolution of the mixtures have been successfully accomplished by using four techniques namely, ^{1}D (first derivative), ^{1}D ratio, CLS (classical least square) and PCR (principal component regression). Variable results were obtained in the different cases as explained in the core of this thesis.

Validation parameters such as calibration range, LOD, LOQ, precision and accuracy parameters (Between F- and t-values respectively) were calculated and a comparison between all the methods and some official methods were done.

The results showed that the PCR technique was the most suitable for analysis of such binary mixtures or the commercial formulations, more than the other techniques and agrees well with those results obtained from the official method.

Key words: chemometrics, derivative spectrophotometry, CLS, PCR, pharmaceuticals, white field ointment , luna soap, and dorin emulsion.

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1. INTRODUCTION

Drug analysis has an important role in the development of medicine. Various analytical and instrumental methods are quite familiar in this area. The great advancement of analytical chemistry is the cornerstone in this field.

The tedious, laborious, costly and time taking process of chemical, analytical and instrumental analysis has become more advanced from time to time. Chromatographic techniques such as: thin layer chromatographic (TLC), high pressure liquid chromatography (HPLC), gas chromatography (GC), etc. have played a pronounced role in drug analysis. The spectroscopic techniques such as ultraviolet-visible (UV-Vis) spectroscopy, fluorescence spectroscopy, infrared (IR) spectroscopy, etc. are now solving qualitative and quantitative problems in chemical analysis.

The trend in analytical chemical analyses has become changed from time to time. One of the instruments being used with excellent precision is UV-Vis spectroscopy [1]. In using the case where significant overlapping of the spectra of mixtures, utilizing this instrument traditionally is hardly possible.

In order to resolve this problem and get more data a computer (software) assisted methods called chemometric is merged in drug analysis [2-5]. The combined use of spectrophotometry and multivariate calibration techniques become methods of choice for the development of better analytical procedures and quality control of many pharmaceuticals [6].

1.1. Chemometric

Chemometric is a field which is related with various disciplines. It is used as a guide to the chemist in extraction of maximum chemical information from complex observations [7].

The following definitions and explanations are asserted by different people. The name chemometric can be divided into *chemo* (from chemistry) and *metric* (meaning measurement). Chemometric thus deals with chemical data and how to obtain information from it [4]

Chemometric is the chemical discipline that uses mathematical and statistical methods to relate measurements made on a chemical system to the state of the system, and design or select optimal measurement procedures and experiments [8].

The following relaxed definition shows also multidirectional aspects of chemometric. *Chemometric is an interdisciplinary field that combines statistics, mathematical methods, computer science and analytical chemistry to solve multivariate problems of data analysis* [9]. The Encyclopedia of Analytical Chemistry, and others also agree to this definition [10-13].

The above aforementioned approach can be expressed diagrammatically as follows:

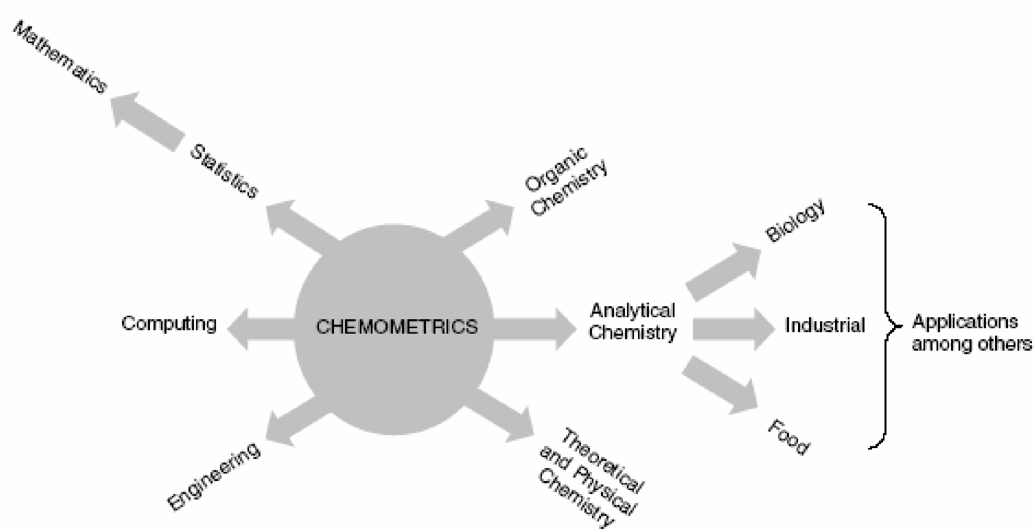


Fig.1. How chemometrics relates to other fields

Chemometric mainly focuses on the chemical mode rather than random effects as is typical for statistics. A chemometric approach does not exclude theory or human experience from problem solving. They fell how to define the problem, what to measure and how to pre-process measurement data, and finally how to interpret the model parameters and the results.

Typical chemometrics strategy comprises:

Collection of data for known cases, generation of a mathematical model which is usually based on multivariate statistics or neural networks, interpretation of the model parameters in terms of the underlying chemistry, application of the model to new cases, and realizing how a meaningful calculations have to be performed [11,13].

To sum the above mentioned explanation, other chemometricians believe that the chemometric basically lie on-collecting and extracting maximum (optimum) information.

Using chemometrics method so many studies were conducted and reported in pharmaceutical science, water quality assessment, sediment-quality analysis, forssenic discrimination, biochemistry, and agricultural chemistry etc. using chemometric assisted techniques [15-19].

1.2. Multivariate analysis

Multivariate analysis is synonymous with the term *chemometric* [10]. It is a method which takes into consideration many variables acting together. The method is fast and efficient in determination as well as extraction of information [20- 22]. Multivariate analysis, as the name suggests, involves looking simultaneously at the relationship between multiple data values, rather than at individual ones [23].

1.2.1. Multivariate calibration

Chemometrics give emphasis on calibration [24]. In calibration the relationship between signals (e.g. absorbance) and mass (or concentration) is set and established very well. The well established relationship, of known concentrations, enables to extrapolate and converts the signals observed (responses) to concentration (for the unknown one). This operation is vital in the analytical laboratories [25, 26].

The term calibration can be defined as the use of empirical data and prior knowledge for determining how to predict unknown quantitative information Y from available measurements X, via a mathematical transfer function. Calibration hence is described as the process of establishing this mathematical function (f) between measured variable x and a dependent variable.

$$Y: f(x) = y \ldots\ldots\ldots\ldots\ldots\ldots [1]$$

One of the simplest forms of calibration is linear regression expression.

$$Y = a+bx.................. [2]$$

where *b* is the regression coefficient and *a* is the intercept of the linear approximation (mass parameter), X is the independent variable and Y is the dependent variable (response parameter). In linear regression one X- variable and one Y- variable are used. In multivariate calibration, however, numerous X and Y variables are used [4].

According to different researchers and the literature, multivariate calibration;

- Used for analysis of large number of samples
- It is the principal cornerstone of chemometrics
- Used to treat complex calibration matrixes
- Used to construct mathematical models at more than one wave length
- A method which has being used widely for quantitative determinations [27-29].

1.2.2 Multivariate calibration Vs Univariate calibration

Multivariate calibration methods have got numerous advantages over univariate calibration methods. Some of these advantages are:-

A. Handling interferents: - unlike in univariate calibration, it does not need to remove background correction. This means that the contribution from one does not affect the contribution from the other. This is the best straight forward advantage of multivariate analysis [30-32].

B. Selectivity: - univariate calibration works well as long as no other components in the sample analyzed absorb light at the wavelength used, i.e. the wave length is selective for the compound under study. If this is not the case, all the interferences in the sample must

be known. In multivariate calibration, however, this is not the case since using many x-variables automatically corrects for each other's selectivity problem, and the x-variables used thus do not need to be totally selective [4,30,32].

C. Outliers control: - multivariates are important to detect outliers in all data analysis. Errors are the rule rather than the exception due to for instance trivial errors, instrument errors and

sampling errors. If these errors are sufficiently large either in quantity or quality, they can affect any meaningful result or interpretation. It may seem difficult to detect outliers when complicated multivariate data are used, but in fact, the detection of outliers is greatly enhanced from having multivariate data [30].

D. Robustness:-Multivariate calibration is more robust to small changes in the experimental or instrumental parameters such as small changes in P^H, temperature or lamp intensity [4]. Generally multivariate calibration has many advantages over the univariate calibration method, with respect to the different analytical parameters.

1.2.3. Multivariate and spectroscopy

There is a close relationship between multivariate analysis method and spectroscopy. This will be explained in detail as follows:

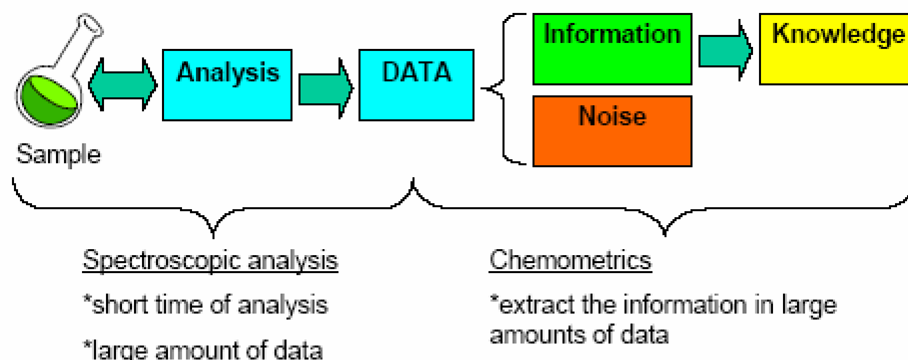


Fig.2. Illustration of why spectroscopy and chemometrics work well in conjunction

Spectroscopic techniques are generally fast, with the analysis from a few seconds to a few minutes and also produce large amounts of data for each sample analysed. Roughly speaking, this data can be said to consist of two parts: information and noise. The information part of the data is what eventually leads to knowledge about the sample, while the noise is a non-information part. A matter of concern is always to minimize and, if possible, to get rid of disturbing noise in the data since it impairs the information gained. This is where chemometrics comes in, since multivariate methods are constructed to extract the information

from large sets of data. Using multivariate data with many variables instead of univariate data offers many advantages in qualitative and quantitative spectroscopic analysis. The methods generally become more robust, precise and less sensitive to background interferences.

One could therefore say that multivariate methods are the optimal choice for the evaluation of spectroscopic data and that the conjunction of spectroscopic analysis techniques with multivariate data analysis offers further possibilities in analytical chemistry.

Multivariate calibration thus means using many variables simultaneously to quantify one or many target variables Y. A calibration model is determined from a set of samples of known content of the calibration set. This can be done by means of PLS or PCR and the resulting model is used to predict the content of new unknown samples from their digitized spectra. The calibration set could consist, for instance, of *m* samples of known content (y). From these samples the n spectral variables are measured. This can be illustrated in figure below.

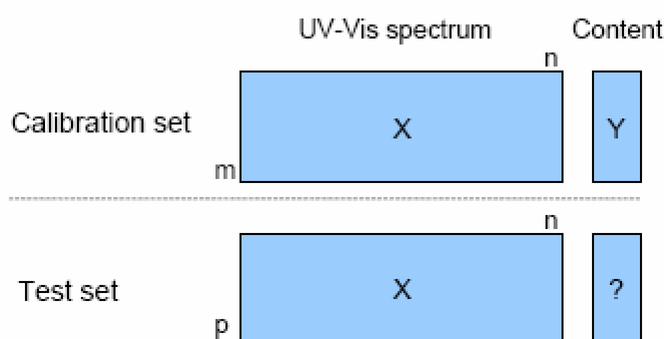


Fig.3. Schematic description of the calibration set and test set used in multivariate calibration.

From the X and Y matrices of the calibration set, the calibration model is then constructed and subsequently validated. The best way to perform this validation is by using new samples not previously used, an external test set consisting of new samples (p) from which the same variables have been measured. By predicting the Y values of the samples in the external test set and then comparing the results with the true values, an estimate of the predictive ability of the model is obtained [4].

As reports reveal, there is advancement in the applications of multivariate analysis in pharmaceutical process development such as: drug discovery, pharmaceutical formulation and quality control of drugs in mixture (two or more drugs) with overlapping spectra [33, 34].

1.3. Multivariate methods

There are various chemometric or multivariate methods. All these methods commonly share the basic principle in multivariate analysis. They are used to resolve the problem in analyzing multi component mixture by allowing rapid and simultaneous determination of each component in the mixture. The accuracy and precision is achieved without prior separation of the components. This implies that these methods are time and cost effective. This will be asserted more as follows:

The resolution of multi component preparations is often a complex analytical problem since combined substances may have different chemical structures but similar properties, like chromatographic behaviour and UV spectra. Multivariate calibration is a useful tool in analysis of multi component mixture because it allows the rapid and simultaneous determination of each component in the mixture with minimum sample preparation, responsible accuracy and precision and without the need of lengthy separation [35].

Although various methods are there in application, three of them will be treated here for the convenience of the study.

1.3.1. Classical least squares regression analysis (CLS)

CLS is one of the traditional regression algorithms, that depends on the Beer Lambert law with assistance of close tools mixtures of components with overlapping spectra will be resolved. Beer's law describes the relationship between two variables, the spectral response (A) and the constituent concentration (c), and two constants, the intercept *(a)* and the regression coefficient *(b)*

$$A_{\lambda 1} = CaKa\,\lambda_1 + C_bK_b\,\lambda_1 \quad \text{[3]}$$

According to beer's law absorbance of multiple constituent at a given wave length is additive.

$$A_{\lambda 2} = C_aK_{a\,\lambda 2} + C_bK_{b\,\lambda 2} \quad \text{[4]}$$

$$A_{\lambda 1} = C_aK_{a1} + C_bK_{b1} \ldots C_nK_{n\,\lambda 1} + E_{\lambda 1} \quad \text{[5]}$$

$$A_{\lambda 2} = C_aK_{a2} + C_bK_{b2} + \ldots C_nK_{n\,\lambda 1} + E_{\lambda 2} \quad \text{[6]}$$

Solving the equations for the K matrix one can use the resulting best fit least squares lines (S) to predict concentrations of unknown analyte [36].

A. Advantage of CLS

- Based on Beer's law
- Unlike to other techniques calculations are relatively simpler,
- The CLS method can be applied for moderately complex mixtures such as binary and ternary mixtures.
- The calibration method does not need the selection of wavelengths necessarily. Once the number of wavelengths to be used exceeds the number of constituents, any number can be utilized, even up to the entire spectrum.
- Making use of large number of wavelength results CLS in giving an averaging effect to the solution. This further leads to less susceptibility to noise in the spectra.

B. Disadvantage of CLS

- It needs understanding the entire composition (i.e. concentration of all constituent) of the mixtures in the calibration mode.
- The method is not applicable for which chemically interact with in mixture
- It is exposed or susceptible to base line effects [36].

1.3.2. Principal component regression analysis (PCR)

It is a factor analysis method. Problems which usually are not solved by traditionally regression methods will be solved better with PCR. It is a well pronounced and known method. As a procedure the steps to be followed are two.

Step 1: Linear combination of the original variable will be combined to optimize a certain criterion. The explained variations in the data are also called latent variables. In short terms no correlation is needed between regression models.

Step 2: In the second step, MLR (multiple linear regression) is applied to the newly obtained latent variables. When co linearity between original variable occurs, interpretation of the variation is observed in the data set than plots of original variable selected by MLR.

A. Advantages of PCR

- PCR doesn't need the selection of wavelength most of the time the whole spectrum are used
- Averaging effect: as one uses great number of wavelengths the averaging effect will be attained decreasing the chance for spectral noise can be utilized for mixtures with large constituents (highly complex). PCR also enables, some times to figure out samples with constituents which are not present basically (originally) in the calibration mixture.

B. Disadvantage of PCR

- The calculation is slower if compared to CLS.
- Optimization needs knowledge of PCA i.e. interpretation and understanding the model is not a simple task.
- It needs large number of samples for the accurate calibration [32,37].

Insipite of the above inconveniences, PCR has widely been applied for the specrophtometric resolution of mixtures comprising two or more serous overlapping spectra [38,39].

1.3.3. Partial least squares

PLS is one of the factor analysis methods that widely used and gives complex information [12, 40]. It is a powerful multivariate statistical tool applied to simultaneous spectrophotometeric determination of mixture with overlapping spectra (of constituents). PLS is the method that is often used in multivariate calibration. It resembles PCR, but works in one step. It also determines latent variables, that are linear combination of the original variables, but the criterion applied is maximal covariance between the Y-values and the spectral variables because of this criterion the algorithm yields models by and interactive procedure which is perceived by the user as a single regression step [39].

A. Advantage of PLS

- It works in one step unlike the PCR
- Can be used for very complex mixture

- Calibrations are generally more robust provided that calibration set accurately reflects range of variability expected in unknown samples
- Combines the full spectral coverage of CLS with partial composition regression of inversed least square [41].

B. Disadvantage of PLS

- The calculations are slower than classical methods
- Models are more abstract, thus more difficult to understand and interpret
- Generally, a large number of samples are required for accurate calibration
- Collecting calibration samples can be difficult and must avoid collinear constituent concentration [41].

From several methods one can use the best model which fits the calibration. It is also possible to use two or more methods to attain comparable results. The following is notified how to select the methods.

Selection of the proper method:

After the preliminary investigation of data structure and according to the considerations given above, one may need to take the correct decision. The following decision scheme may help the analyst to select the proper multivariate method to be used:

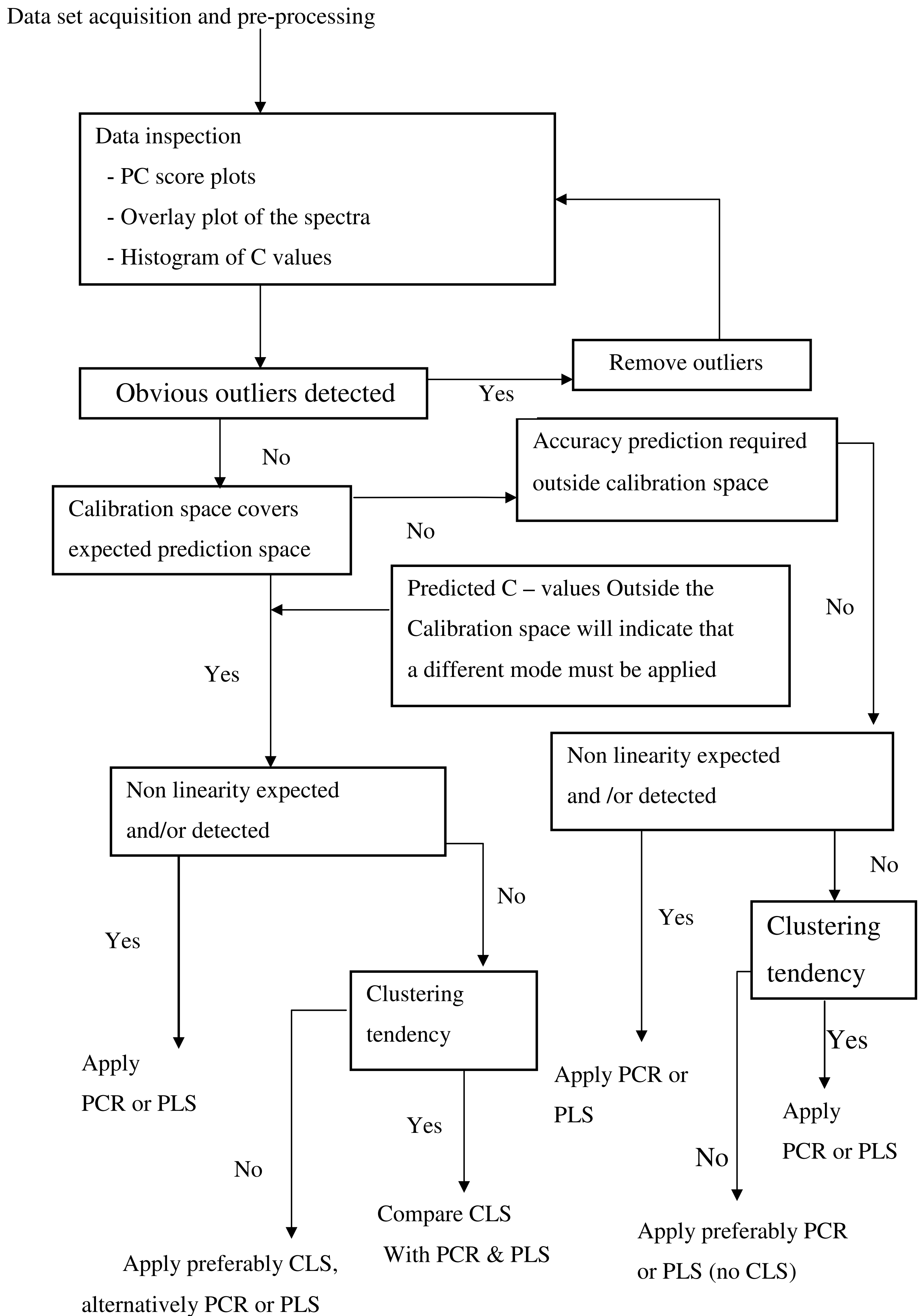


Fig.4. Decision scheme for selection of the proper multivariate method

The advent of rapid, in expensive computers has permitted the proliferation of computationally intensive calibration methods. One must choose between many competing methods and algorithms for application to any particular calibration challenge. Although it is well known that certain calibration method is not applicable in some situations, it is often a daunting challenge for many calibration method and users to decide with confidence as which calibration method situation. The need to develop logical rules to aid in calibration method selection is imperative since, as technology progresses, technologists are moving closer to implementing multivariate and higher order sensors capable of self calibration. The best calibration method for future prediction of accuracy and precision will be the one that employs the simplest model that fits the calibration data. If otherwise equivalent methods are required to model the data to arbitrary precision, the method that incorporates a basis set that best mimics the data will construct the simplest model [42].

1.4. Derivative spectrophotometry (DS)

Derivative spectrophotometry is one of the analytical techniques used for extraction of quantitative and qualitative information from unresolved spectra bands by making use of the first or higher derivatives. It comprises the differentiation of a normal spectrum in order to enhance the resolution of mixtures by increasing the detectability of minor (weak) features towards readily measured maxima.

The techniques have a better way for the improvement of sensitivity and specificity within the analysis of mixture when the constituents are with very similar spectra [43].

The two similar compounds can be distinguished much more readily from the derivative than the normal spectra [44]. Because of this a great attention is taken towards derivative spetrophotometry. Mathematically the derivative spectra are expressed as follows:

Zero order $A = f(\lambda)$ .... [7]

First order $f'(\lambda) = \frac{dA}{d\lambda}$ .... [8]

Second order $\frac{d^2A}{d\lambda^2} = f''(\lambda)$ .... [9]

If Beer's Law is satisfied for the zero-order spectrum the relationship between concentration and amplitude can be shown as:

Zero order $A = \in bc$......................... [10]

First order $\frac{dA}{d\lambda} = \frac{d \in bc}{d\lambda}$.................... [11]

n^{th} order $\frac{d^n}{d\lambda^n} = \frac{d^n \in bc}{d\lambda^n}$.................. [12]

Where A is absorbance, $\in$ is the extinction coefficient, b is the sample path length and C is the sample concentration [45].

Many types of analyses would be improved by using this principle because the use of an absorbance spectrum is no more difficult than the use of derivative spectrum. The amplitude between the minimum and maximum on the nth derivative curve is more proportional to the values of the absorbance of the solution. A calibration curve can be obtained from several standard solutions of varying concentrations to which the same mathematical principles are applied [46].

1.4.1. Characters of Derivative Spectrophotometer

1. Increase of spectra resolution

The most prominent feature of DS is the improvement of the resolution of overlapping spectral bands. It gives due attention to sharp features of a spectrum at the expense of broad bands [45, 47, 48].

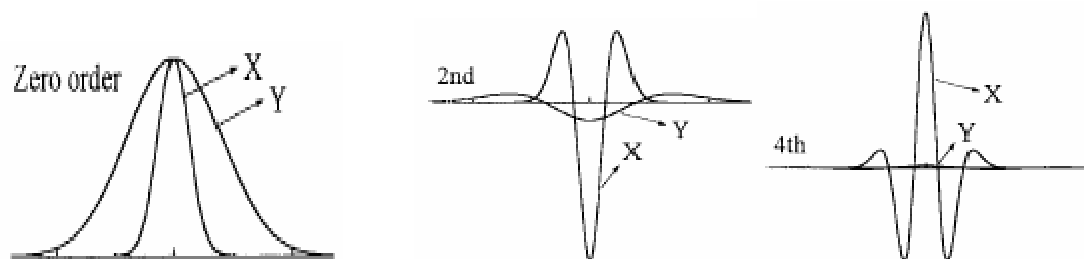


Fig.5. Effect of derivative order (zeroth, second and fourth) on the relative amplitudes of two coincident Gaussian bands, X and Y, of equal intensity but with a band- width ratio 1:3 [47].

The derivative spectra are always more complex than the zero-order spectrum. The first derivative is the rate of change of absorbance against wavelength. It starts and finished at zero, passes through zero at the same wavelength as λmax of the absorbance band with first a positive and then a negative band, with the maximum and minimum at the same wavelengths as the inflection points in the absorbance band. This bipolar function is characteristic of all the odd-order derivatives. The most characteristic feature of the second-order derivative is a negative band with the minimum at the same wavelength as the maximum on the zero-order band. It also shows two additional positive satellite bands on either side of the main band. The fourth derivative shows a positive band. The presence of a strong negative or positive band, with the minimum or maximum at the same wavelength as λmax of the absorbance band is characteristic of the even-order derivatives. Note that the number of bands observed is equal to the derivative order plus one [43].

2. Elimination of the influence of baseline shift and matrix interferences

Qualitative and quantitative investigations of broad spectra are frequently difficult, especially where the measurement of small absorbencies is concerned, because of uncontrollable baseline shift, great blank absorption and matrix interferences, regardless of whether they are caused by irrelevant absorption of light scattering by turbid solutions and suspensions. All these influences can be overcome by derivatisation. The order of derivatisation depends on the order of the polynomial function used to describe interferences.

3. Enhancement of the detectability of minor spectra features

The derivative spectrophotometer amplifies the weak variations in the slopes of the initial spectrum for better detection [46, 47].

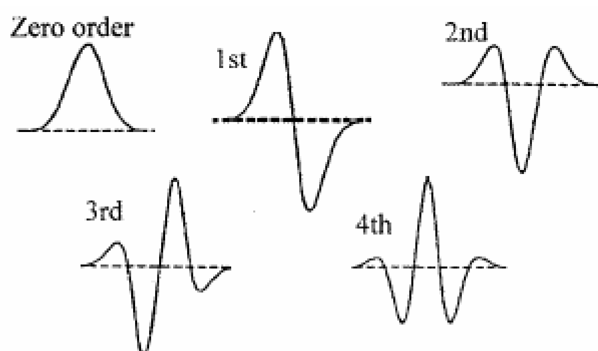


Fig.6. Characteristic profiles of derivative orders of a Gaussian band.

4. Precise determination of the positions of absorption maxima

When a single-peak spectrum has a broad band as its main feature, the position of the absorption maximum can be only approximately determined. The first derivative of this band ($dA/d\lambda$) passes through zero at the peak maximum, minimum and shoulder points (Fig.6) and can be used to accurately locate the peak position. In contrast, the second and higher even derivatives ($d_2A/d\lambda_2$, $d_4A/d\lambda_4$,...) contain a peak of changeable sign (negative in the second order, positive in the fourth order, etc.) which has the same position as a peak maximum in the normal spectrum. The width of this peak progressively decreases with increasing order of the even derivative which causes a sharpening of the peak enabling its exact identification. However, every even derivative peak is accompanied by symmetrical satellites of the opposite sign, the number of which is equal to the derivative order. In higher order derivatives ($d_2A/d\lambda_2$, $d_4A/d\lambda_4$ ...) the satellites of adjacent bands may interfere, thus limiting the observed resolution. Also, during differentiation of synthesized spectral profiles peaks of certain components might be shifted compared to their original positions [47].

5. Signal-to-noise ratio (SNR)

The major disadvantage of derivative spectrophotometry is that the level increases in the derivative spectrum. This worse condition can be improved by summing and overlapping spectra [45, 47].

The Ds can be obtained by optical, electronic or mathematical methods. Optical and electronic techniques were used on early UV/Vis spectrophotomers but have largely superseded by mathematical techniques. The advantages of the mathematical techniques are that derivative spectra may easily be calculated and recalculated with different parameters, and smoothing techniques may be used to improve signal-to-noise ratio [43].

1.4.2. Derivative Ratio Method

Absorbance ratios have been used by the British pharmacopoeia for the identity and purity of certain pharmaceuticals. The use of absorbance ratio spectra has been the basis of some analytical procedures. The method is based on the use of each component in turn as a reference standard and provided an over-dimensioned system that can only be solved graphically.

The method is based on the use of the first derivative of the ratios of spectra. The absorption spectrum of the mixture is obtained and the amplitudes at appropriate wavelengths are divided by the corresponding amplitudes in the absorption spectrum of a standard solution of one of the components. The first derivative of the ratio spectrum is obtained. The concentration of the other component is then determined from a calibration graph.

The first derivative of the ratio spectra method resolves many binary mixtures which can not be solved by the application of the normal derivative technique; the method still has some pit falls. The method shows some limitations because in the wavelength range where the absorbance of the standard solution used as divisor is zero (or below the base line) the noise is strongly exalted. In consequence, the useful wavelength range must be selected and, if noise is now slightly exalted, a smoothing function (prior to derivation) can be used in classical derivative spectrophotometry.

1.4.3. Multi-component analysis

The accuracy of the result obtained in multi-component analyses by application of multi-variety calibration to the absorbance signals depends on the particular method and analytical signal used [43]. This, Ds, methods is used for the calibration matrices such as PLS, PCA are utilized.

1.5. Benzoic acid, salicylic acid and resorcinol

A. Benzoic Acid

Character: A white, crystalline powder or colorless crystals, odorless or with a very slight characteristic odor, slightly soluble in water, soluble in boiling water, freely soluble in alcohol, in ether and in fatty oils.

Action and use: Antimicrobial, preservative, usually used in combination with salicylic acid in ointments.

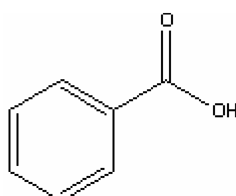


Fig.7. Chemical structure of benzoic acid

B. Salicylic Acid

Character: A white, crystalline powder or white or colorless, acicular crystals, slightly soluble in water, freely soluble in alcohol and in ether, sparingly soluble in methylene chloride.

Action and use: Keratolytic.

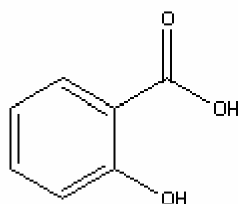


Fig.8. Chemical structure of salicylic acid

White field is an ointment which contains 6.0% w/w of benzoic acid and 3.0% w/w of salicylic acid in a suitable ointment base. It combines the fungicidal action of benzoic acid and keratolytic action of salicylic acid. It is often prescribed for epidermophytosis and ring worm of the scalp since benzoic acid is fungicidal and eradication of the infection occurs only after the infected stratum corneum is shed and continuous medication is required for several weeks. Its keratolytic action makes salicylic acid a beneficial agent in the local treatment of fungus infections and certain form of eczematoid dermatitis.

Benzoic acid and salicylic acid has similar UV spectra. In mixture form their spectra is highly overlapping. It is impossible to determine the concentration of both compounds in UV spectrophotometry simultaneously without separations in a univariate method.

C. Resorcinol

Character: a colourless or slightly pinkish-grey, crystalline powder or crystals, turning red on exposure to light and air, very soluble in water and in alcohol, freely soluble in ether.

Action and use: antifungal and antibacterial action

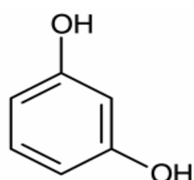


Fig.9. Chemical structure of resorcinol

A ternary mixture of benzoic acid, salicylic acid and resorcinol is used for pharmaceutical formulations. Three of them have very overlapping UV spectra [49, 50].

2. OBJECTIVES

2.1. General objectives

- The study is mainly aimed at analyzing these strongly overlapped *(benzoic and salicylic acid, or salicylic acid and resorcinol)* binary combinations by using chemometrics-assisted spectrophotometric techniques without prior separation of them, and comparing the obtained results with those obtained from official, or reported methods.

2.2. Specific objectives

1. To determine the quantities in binary (benzoic & salicylic acid, salicylic acid & resorcinol) with derivative method.

2. To determine the quantities in binary (benzoic & salicylic acid, salicylic acid & resorcinol) derivative ratio method.

3. To determine the quantities in binary (benzoic & salicylic acid, salicylic acid & resorcinol) with the classical least square (CLS) method and.

4. To determine the quantities in binary (benzoic & salicylic acid, salicylic acid & resorcinol) with principal component regression (PCR) method.

3. EXPERIMENTAL

3.1. Apparatus

Spectrophotometric measurements were carried out on a computerized double beam UV/Visible spectrophotometer (Unicam, England). The absorption spectra of test, and reference solutions were recorded over the range 200-400 nm. The subsequent statistical manipulation was performed by transferring the spectral data to Microsoft Excel program and processing them with the standard curve fit package and matrix calculations.

3.2. Chemicals

Pharmaceutical grade benzoic acid (BDH, England) salicylic acid (BDH, England), and resorcinol (BDH, England), were used as working standards after confirming their purity and compliance with pharmaceutical requirements. All other reagents and solvents used were analytical grade.

3.3. Pharmaceutical preparations

The following pharmaceutical preparations were purchased from the local and Egyptian markets and subjected to analysis by the proposed procedures:

1. Whitefield ointment (EPHARM, Ethiopia) labelled to contain 6 % benzoic acid and 3 % salicylic acid
2. Luna soap (Egypt) labelled to contain 2% salicylic acid and 2 % resorcinol
3. Dorin emulsion (Egypt) labelled to contain 2% salicylic acid and 1 % resorcinol

3.4. Procedures

3.4.1. Preparation of standards

In to 100 ml volumetric flask a weighed amount (50 mg) of either of the standards is dissolved in about 100 ml of 0.1 M sodium hydroxide and diluted to volume with the same solvent. The resulting solution is diluted quantitatively with 0.1M sodium hydroxide to obtain the appropriate dilutions for each drug according to its linear calibration range or as specified under the analysis of the laboratory prepared mixtures.

3.4.2. Preparation of samples

A. Whitefield: From a tube of ointment 0.667 g weighed amount was extracted three times with ether, and then separated using reparatory funnel. The organic layer was washed 3x5 ml with 0.1M NaOH solution. The combined aqueous layer was collected quantitavely and completed to 100 ml volumetric flask with 0.1M NaOH.

B. Luna soap: One gram of the soap was weighed and finely powdered. The weighed amount of the powder is transferred to 100 ml volumetric flask and dissolved with 25 ml of 0.1 M sodium hydroxide, filtered and diluted to 100 ml volumetric flask with 0.1M NaOH. The stock solution is diluted quantitavely with 0.1M NaOH to obtain the suitable working sample solutions for the UV-measurements.

C. Dorin emulsion: One gram of the emulsion was extracted three times with ether, and then separated using reparatory funnel. The organic layer was washed 3x5 ml with 0.1M NaOH solution. The combined aqueous layer was collected quantitavely and completed to 100 ml volumetric flask with 0.1M NaOH.

3.4. 3. Standard solutions for ^{1}D,^{1}D ratio and multivariate calibration

For first derivative analysis ten solutions were prepared each of the pure components (benzoic acid, salicylic acid and resorcinol) with concentrations range from 5-50 μg/ml as described in section 3.4.1. The normal zero-order UV absorption spectrum of each solution as well as the first-derivative spectrum was recorded over 200-400 nm range against a blank solution prepared similarly. After the determination of the zero-crossing wavelengths for the cited drugs, the standard curve for each drug was constructed by plotting the measured amplitudes versus the corresponding drug concentrations:

(1) at 237.5 nm and 310.5nm for benzoic acid and salicylic acid respectively. The values of the first derivative amplitude at 310.5 nm (zero-crossing of benzoic acid) were measured for determination of salicylic acid in presence of benzoic acid and those at 237.5 nm (zero-

crossing of salicylic acid) were used for determination of benzoic acid in presence of salicylic acid.
(2) At 290.5 nm and 317.5 nm for salicylic acid, and 297.5 nm for resorcinol. The values of the first derivative amplitude at 297.5 nm (zero-crossing of salicylic acid) are measured for determination of resorcinol in presence of salicylic acid and those at 290.5 nm and 317.5 nm (zero-crossing of resorcinol) are used for determination of salicylic acid in presence of resorcinol.

For derivative ratio analysis similar concentration range, wavelength ranges and measuring steps were followed as first derivative technique. After recording the first derivative values the first derivative ratio spectrum of each component was obtained by dividing to the corresponding component's derivative spectrum and the effect of divisor and wavelength is observed.

Accordingly ; (1) at 269.5 nm the peak height was taken for benzoic acid with the divisor 25 µg/ml with 3 nm differences. For salicylic acid 292.5 nm and 308.5 nm are taken as amplitude with a divisor of 50 µg/ml benzoic acid at 4 nm differences. (2) at 314.5 nm and 323.5 nm the peak heights were taken for salicylic acid with the divisor 25 µg/ml resorcinol with 3 nm differences. For resorcinol 242.5 nm and 302.5 nm are taken as amplitude with a divisor of 5 µg/ml salicylic acid at 1 nm differences.

In order to confirm the precision of simultaneous determination ten for benzoic acid and salicylic acid combination, and three for salicylic acid and resorcinol laboratory prepared mixtures of the studied drugs in different ratios were prepared and analyzed as described before. The concentration of each drug in the studied mixtures was then predicted using the least square line equation obtained with its pure standard solutions used in the calibration stage.

In order to obtain the calibration matrixes for applying CLS and PCR analysis, ten solutions of each of the pure components (benzoic acid, salicylic acid and resorcinol) were prepared with the same concentration levels mentioned for ^{1}D UV procedure. These ranges were previously verified to obey Beer's law for each of the studied drugs in the selected alkaline solutions. The absorption data in the range 200-400 nm (digitized every 1.0 nm) were subjected to the least squares analysis in order to obtain the calibration matrix K for each

drug (see the discussion section). The laboratory prepared mixtures were then prepared by mixing known amounts of (1) *benzoic acid with salicylic acid*, (2) salicylic acid with resorcinol, in different varied proportions in order to verify the precision of the method for analysis of such mixtures and matching the commercial drugs (mixtures) with those having the same or approximately comparable concentrations.

PCR multivariate analysis follows the same procedure described for CLS except that the K matrix must be replaced by F matrix, which is calculated from the factorized data instead of absorbance values in case of CLS and A matrix must be replaced by $A_{proj.}$

3.4.4. Data processing

Data were processed on an Intel Pentium IV-Version, PC-compatible computer equipped with essential statistical programs for CLS and PCR calculations.

4. RESULT AND DISCUSSION

4.1. Preliminary investigation of data structure in chemometrics techniques

The original laboratory (experimental) observations are taken as a row data and well tabulated systematically for the mathematical, statistical and computer software analysis purpose.

The pure benzoic acid, salicylic acid and resorcinol data are tabulated, discussed and analyzed first (calibration step). Next to pure forms the laboratory-prepared mixtures with different ratios are treated accordingly (testing step). Eventually the dosage form (commercial) mixtures are analyzed (prediction step).

The experiments were done in triplicates in all cases in different three days with the same procedure, laboratory equipment, time and analyst (to confirm both repeatability and reproducibility precisions). Then the mean averages of the triplicates for every day and all the three days are then taken in this analysis. Subsequently various graphs are sketched from the tabulated data using different software such as MS Excel, Harvard Graphics 2.0 and Vista 6.0.
These preliminary graphs may help us in reaching a final conclusion about the data structure and the proper selection of the used analytical method:-

1. Overlay plots of the given spectra:-
This will make apparent gross outliers and clear clusters in the given responses (absorption in our case) Figures 10-12, represent the overlay plots for the standard compounds (benzoic acid, salicylic acid, and resorcinol respectively) at the different concentration levels.

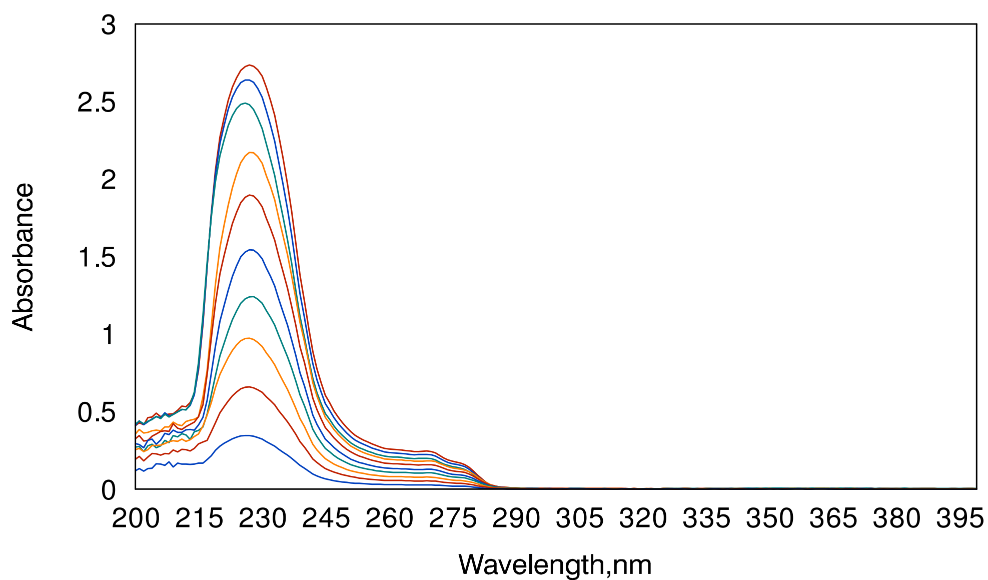


Fig.10. Overlay absorption curves of benzoic acid in the calibration range (5-50 μg/ml).

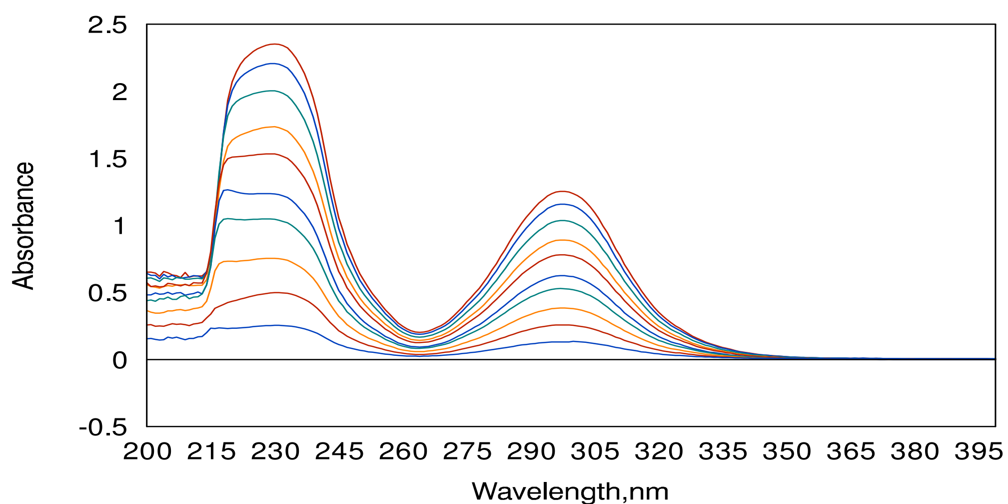


Fig.11. Overlay absorption curves of salicylic acid in the calibration range (5-50 μg/ml).

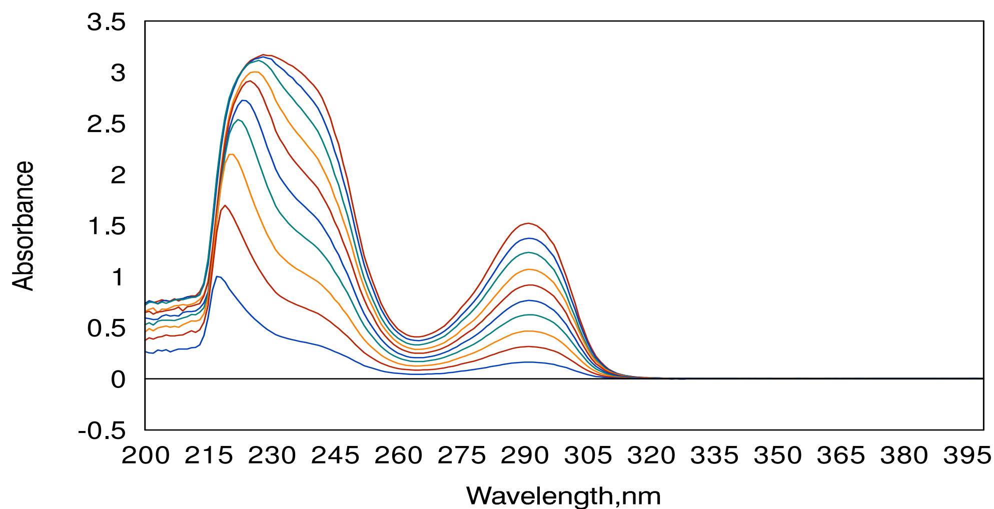


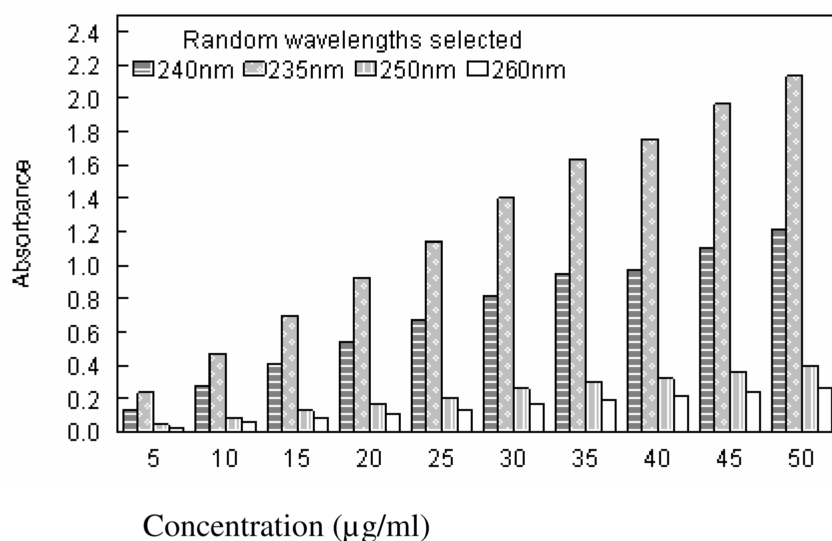
Fig.12. Overlay absorption curves of resorcinol in the calibration range (5-50 μg/ml).

From the three graphs, one can readily observe that no obvious studied overlapping and /or crossing of the spectra lines are observed. The lowest concentration is at inner bottom (5 μg/ml) with shortest amplitude and that of the highest concentration is at the top outside (50 μg/ml) with longest amplitude.

2. A histogram plot of the C-values:

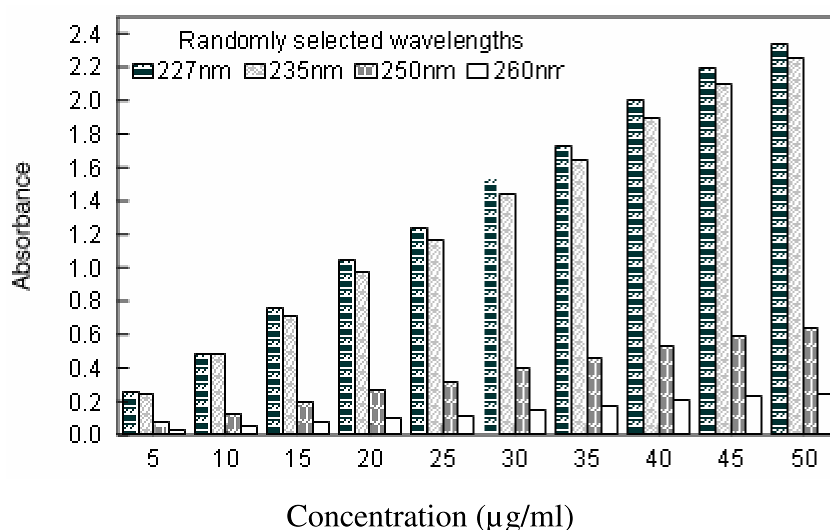
This will make apparent clusters and gross outliers in the concentration gradient (C).

Figures 13-15, represent the concentration dependent histograms for the studied compounds at four randomly selected wavelengths.



Concentration (μg/ml)

Fig.13. The concentration dependent graph (C-histogram) for pure benzoic acid at four randomly selected wavelengths



Concentration (μg/ml)

Fig.14. The concentration dependent graph (C-histogram) for pure salicylic acid at four randomly elected wavelengths

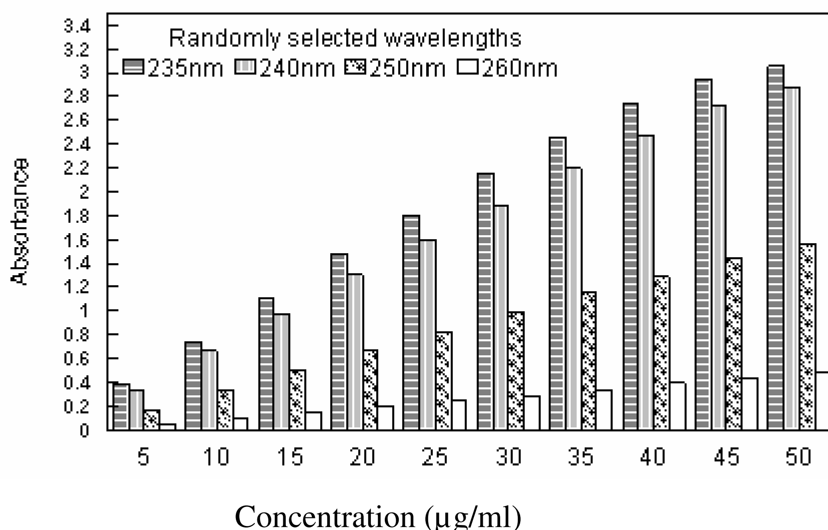


Fig.15. The concentration dependent graph (C-histogram) for pure resorcinol at four randomly selected wavelengths

It is obvious, from the graphs, that in all cases the responses are concentration dependent and there are not any abnormalities or outliers with respect to the C-values. The above figures (13-15) have got good dependable concentration-absorbance correlations that can be in a position to conduct the research further.

3. Relation plots between the principal components (in case of the PCR analysis):-

Figures 16-21, represent the box and scree plots for the three studied compounds and their mixtures

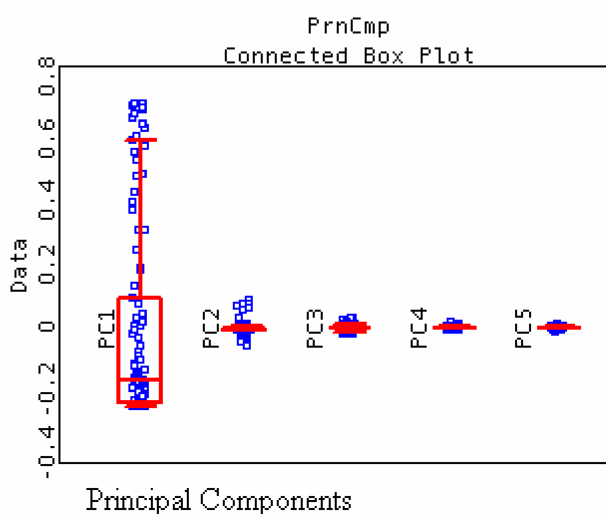


Fig.16. Box plot for pure benzoic acid in the calibration range (5-50 µg/ml)

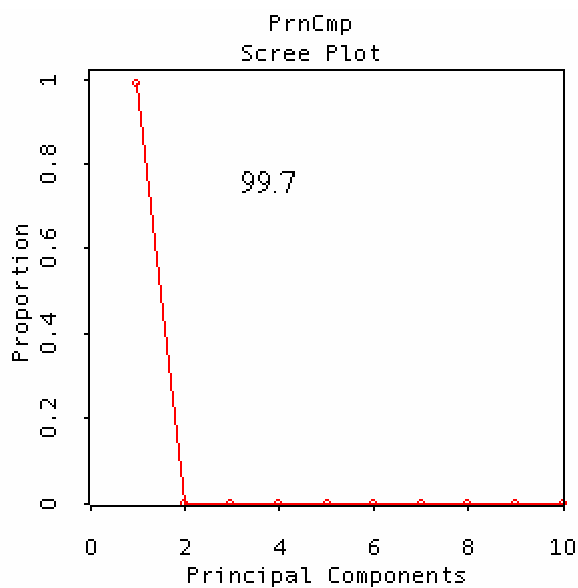


Fig.17. Scree plot for pure benzoic acid in the calibration range (5-50 µg/ml)

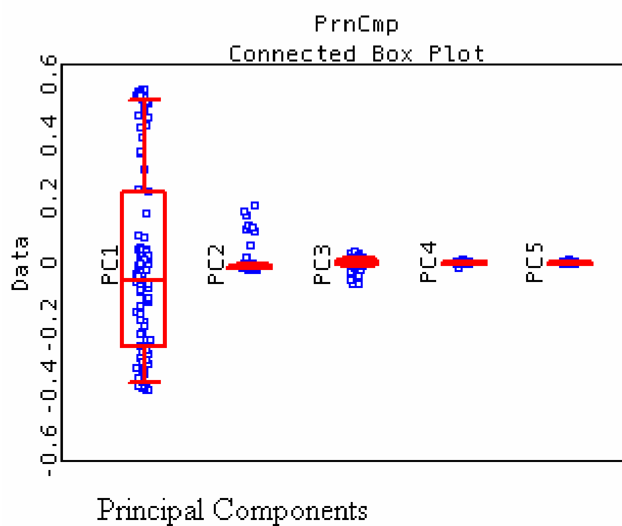


Fig.18. Box plot for pure salicylic acid in the calibration range (5-50 µg/ml)

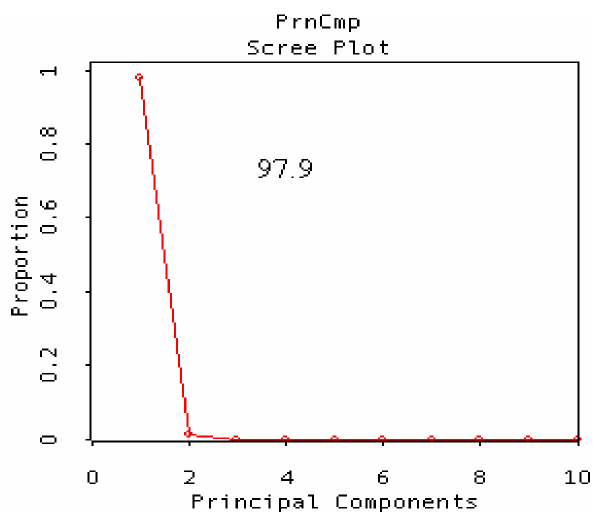


Fig.19. Scree plot for pure salicylic acid in the calibration range (5-50 µg/ml)

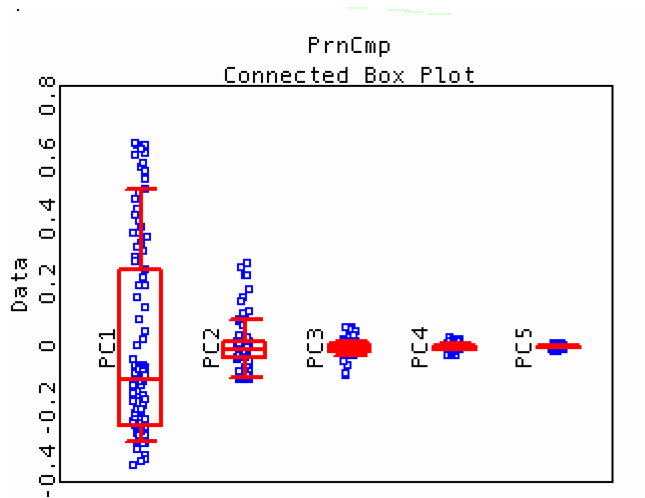


Fig.20. Box plot for pure resorcinol in the calibration range (5-50 µg/ml)

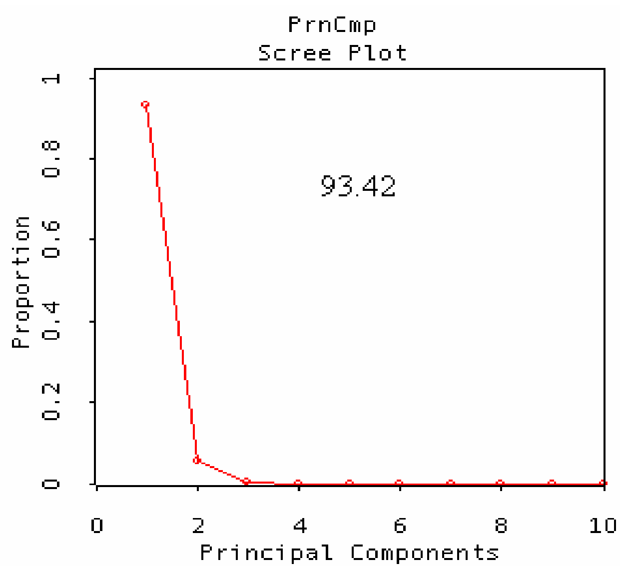


Fig.21. Scree plot for pure resorcinol in the calibration range (5-50 µg/ml)

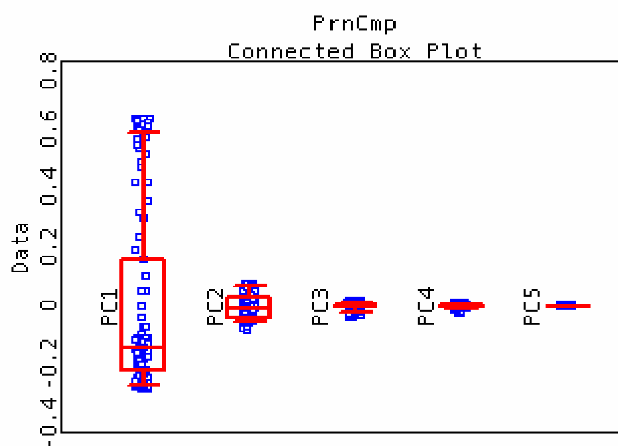


Fig.22. Box plot for benzoic acid with salicylic acid mixture

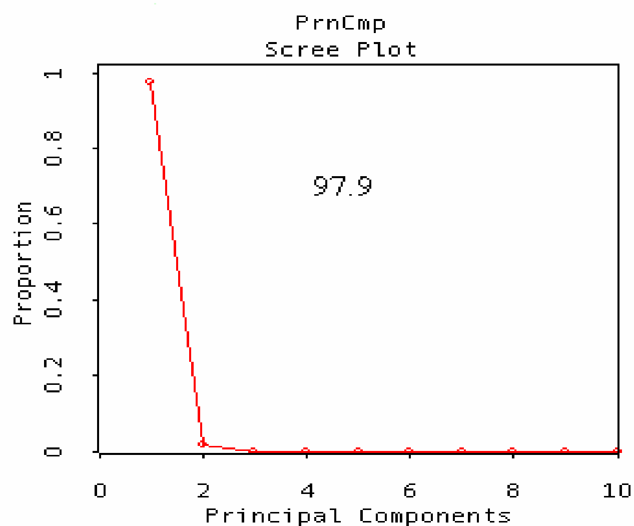


Fig.23. Scree plot for benzoic acid with salicylic acid mixture

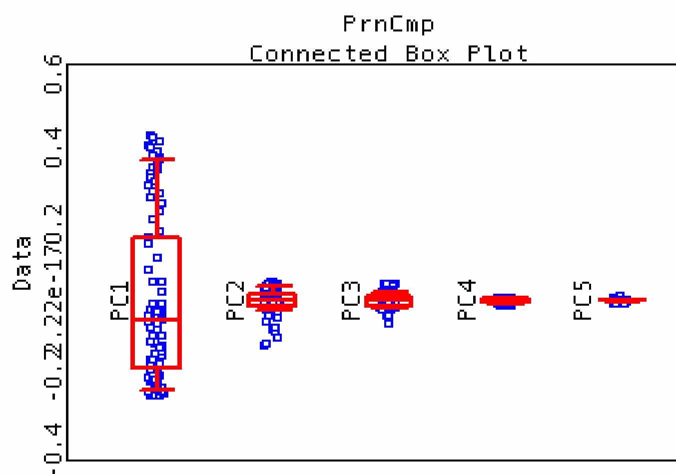


Fig.24. Box plot for salicylic acid with resorcinol mixture

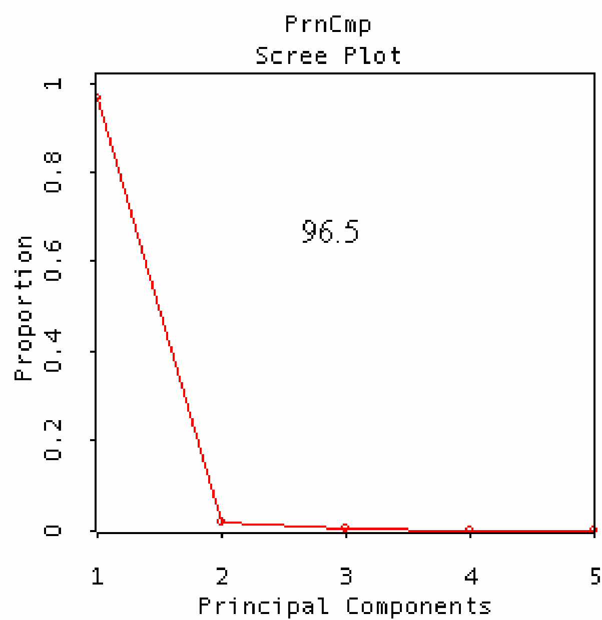


Fig.25. Scree plot for salicylic acid with resorcinol mixture

It is obvious from the figures that the first principal component in all cases is the main one in the calculations (exceeding 92% of the explained data). Strong non-linearities (if any) will probably shown up on one of these plots. It is recommended that, even if one has already decided to use a specific method (for instance, because it is the only one included in the available software), these plots should be obtained. In addition, it should be understood that at this stage no exhaustive search into the presence of non-linearities, clusters and outliers is carried out. Minor non-linearites, clustering and less gross outliers do not need to be detected at this stage.

4. Degree of overlapping:-

As can be seen from figures 26 and 27 considerable degrees of spectral overlapping occur in the region from 215 to 350 nm, for benzoic acid with salicylic acid, and salicylic acid with resorcinol. The degree of spectral overlapping can be conveniently for both binary mixtures given by $(Di)^{0.5}$, where Di is the magnitude dependency that can be calculated for a two components mixture from the equation:-

$$D_i = \frac{(\Sigma K_1 K_2^t)^2}{\Sigma K_1 K_1^t . \Sigma K_2 K_2^t} \text{ .... [13]}$$

Where K_1 and K_2 are L x n matrices of regression coefficients for the constituents of the mixture.

A. Degree of overlapping of benzoic acid with salicylic acid

In the case of the presently studied compounds, as indicated by the spectra found in figure 26 the magnitude of dependency (Di) was found 0.7655 that yielding about 87.5% of spectral overlap between the mixture components.

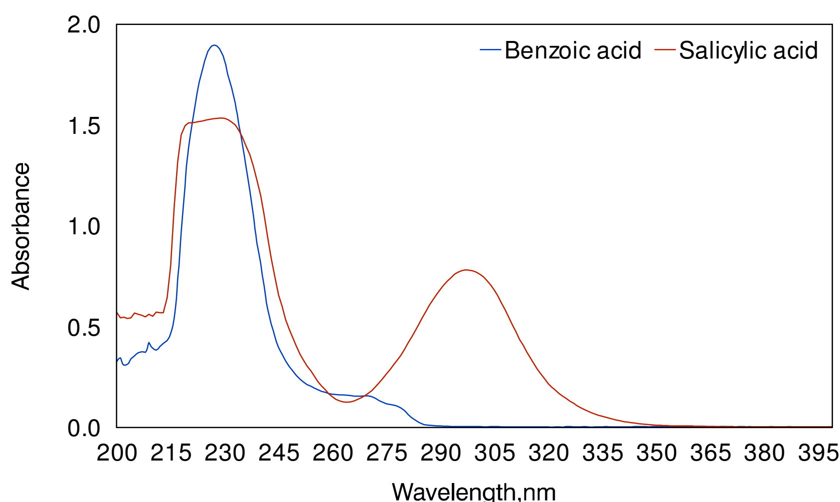


Fig.26. The degree of overlapping between benzoic acid 40 µg/ml and salicylic acid 40 µg/ml

B. Overlapping in salicylic acid and resorcinol mixture

In the case of the presently studied compounds, as indicated by the spectra found in figure 27 the magnitude of dependency (Di) was found 0.9254 that yielding 96.2% of spectral overlap between the mixture components.

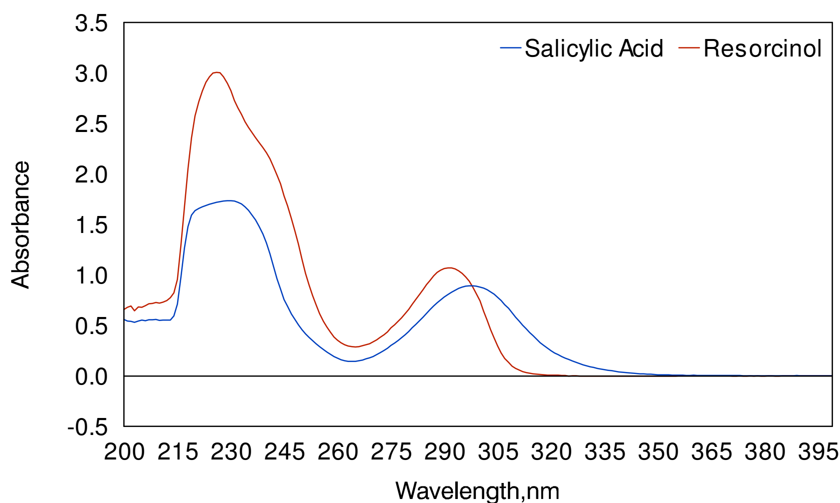


Fig.27. The degree of spectral overlapping between salicylic acid and resorcinol

From figure 27 and the calculated degree of overlapping we observe that the spectrum of salicylic and resorcinol are highly overlapped. Salicylic acid overlaps more with resorcinol than benzoic acid. As observed in the pure compounds salicylic acid and resorcinol have readings near to 300 nm but not benzoic acid.

From the calculated degree of overlapping and as shown in figures 26 and 27, one can conclude that both mixture constituents can not be determined by using the ordinary spectroscopic methods unless prior separation techniques or chemometric techniques are applied.

4.2. First derivative method

A first derivative gives a better resolution of spectra than that of the zero order absorption spectra. The first overlay derivative spectra of the studied drugs separately (figures 28, 29 and 31) or in binary combinations (Figures 30 & 32) show good identified zero-crossing points that can be used for simultaneous determination of the studied drugs.

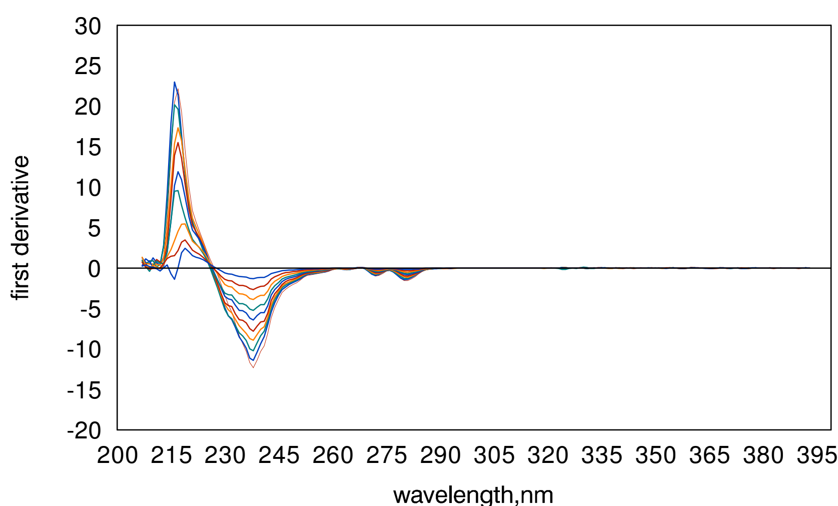


Fig.28. First derivative spectra of pure benzoic acid at different concentration levels (5-50 µg/ml)

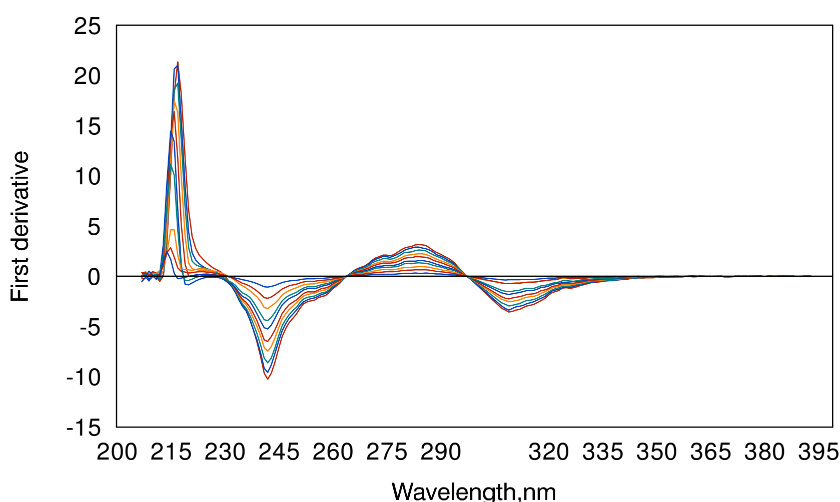


Fig.29. First derivative spectra of pure salicylic acid at different concentration levels (5-50 µg/ml)

4.2.1. Benzoic acid- salicylic acid mixtures

The wavelength 237.5 nm was used for determination of benzoic acid in presence of salicylic acid (zero crossing wavelength of salicylic acid) and the wavelength of 310.5 nm was selected for estimation a salicylic acid in presence of benzoic acid (zero crossing wavelength of benzoic acid). The wavelengths selected are those exhibiting the best linear responses. The linear relationships between the derivative amplitudes as drug concentration were obtained over the concentration range of 5-50 μg/ml for both drugs.

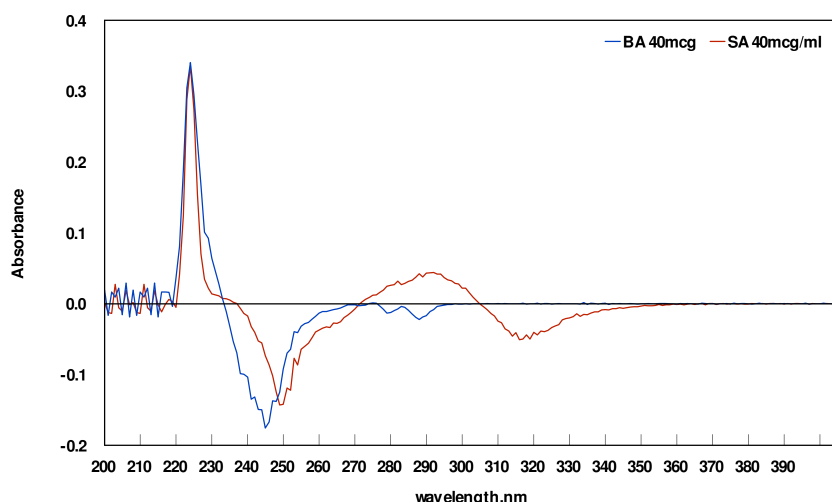


Fig.30. First derivative spectra for benzoic acid (40 μg/ml) and salicylic acid (40 μg/ml) indicating the zero-crossing points of each of them

By taking the zero-crossing points of salicylic acid for the estimation of benzoic acid, we obtained the analytical parameters and predicted values presented in tables (Table 1 and Table 2). The same manner was conducted for salicylic acid determination and the results of it are presented in tables 1 and 3.

Table 1. Spectral and analytical parameters for both benzoic acid and salicylic acid as calculated from the ^{1}D zero-crossing points against the given concentrations of standard compounds at the selected wavelengths:

Compound	λ* (nm)	LC R ** (µg/ml)	Slope (b) ±SE***	Intercept (a) ±SE	r *	r^{2} **	LOD† (µg/ml)	LOQ†† (µg/ml)
Benzoic acid	237.5	5-50	0.00058±0.00252	-0.00421±0.00008	0.9985	0.997	1.8	6.0
Salcylic acid	310.5	5-50	-0.00029±0.00045	-0.00107±0.00002	0.9993	0.999	1.3	4.2

* λ=wavelength of determination, ** LCR= linear calibration range, *** SE=standard error, * r= correlation coefficient

** r^{2}= determination coefficient, † LOD= limit of detection, †† LOQ= limit of quantification

Table 2. Actual and predicted amounts of benzoic acid given by applying first derivative spectrophotometric technique for pure, laboratory prepared mixtures with salicylic acid and white field ointment (determined at 237.5 nm).

Component analyzed	Concentrations used (µg/ml)	Recovery		
		µg/ml	%	* RSD
Pure benzoic acid	10	10.14	101.4	0.7
	15	14.70	98.0	3.0
	20	19.88	99.4	2.1
	25	24.50	98.0	1.9
	30	29.28	97.7	4.3
	35	35.11	100.3	2.4
	40	41.83	101.6	0.9
	45	45.51	101.1	3.2
	50	48.76	97.5	0.5
benzoic acid in binary mixture with salicylic acid	50(10)*	49.77	99.5	0.7
	45(15)	44.73	99.4	2.0
	40(20)	40.34	100.9	2.9
	35(25)	35.70	102.0	0.4
	30(30)	29.70	99.0	2.6
	25(35)	25.26	101.1	1.4
	20(40)	19.60	98.0	0.7
	15(45)	15.19	101.3	1.4
	10(50)	10.19	101.9	0.9
Whitefield ointment	40(20)	40.06	100.2	1.3

* ( ) the concentrations of salicylic acid in the studied mixtures in µg/ml

* RSD is the relative standard deviation

Table 3. Actual and predicted amounts of salicylic acid given by applying first derivative spectrophotometric techniques for pure, synthetic mixtures with benzoic acid and commercial dosage forms white field ointment (determined at 310.5 nm).

Component analyzed	Concentrations used (μg/ml)	Recovery		
		μg/ml	%	*RSD
Pure salicylic acid	10	9.84	98.4	1.2
	15	14.82	98.8	0.9
	20	20.73	100.4	3.1
	25	24.30	97.2	1.2
	30	30.18	101.0	0.4
	35	35.17	100.0	0.4
	40	40.88	102.0	1.4
	45	45.26	101.0	0.4
	50	49.00	98.0	0.1
Salicylic acid in binary mixture with benzoic acid	10 (50) *	10.07	100.7	0.5
	15 (45)	14.84	98.9	0.8
	20 (40)	20.27	101.4	0.9
	25 (35)	25.48	101.9	1.3
	30 (30)	30.13	100.4	0.3
	35 (25)	34.10	97.4	1.9
	4 (20)	40.49	101.2	0.9
	45 (15)	44.92	99.8	0.1
	50 (10)	48.98	98.0	1.5
Whitefield ointment	20 (40)	19.85	99.3	0.5

* ( ) the concentrations of benzoic acid in the studied mixtures in μg/ml

* RSD is the relative standard deviation

4.2.2. Salicylic acid – resorcinol mixtures

The wavelength 290.5 nm and 317.5 nm were used for determination of salicylic acid in the presence of resorcinol (zero-crossing wavelengths of resorcinol) and the wavelength of 297.5 nm was selected for estimation a resorcinol in presence of salicylic acid (zero-crossing wavelength of salicylic acid).The wavelengths selected are those exhibiting the best linear responses. The linear relationships between the derivative amplitudes as drug concentration were obtained over the concentration range of 5-50 μg/ml for both drugs.

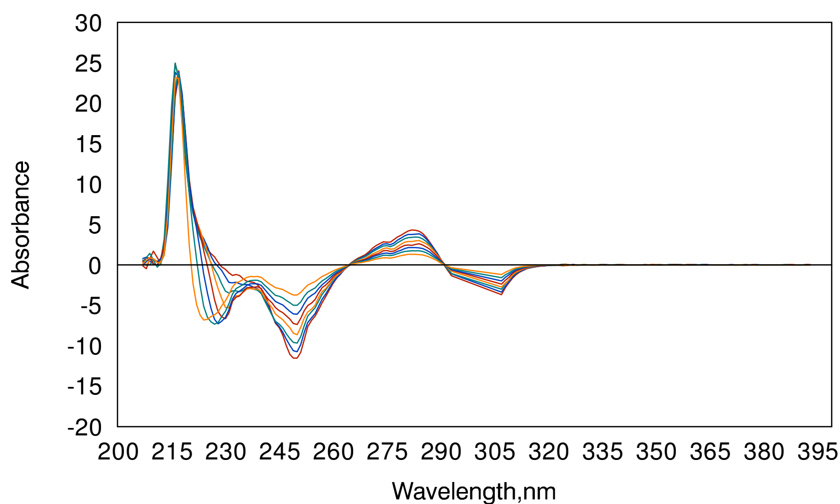


Fig.31. First derivative spectra of resorcinol at different concentration levels (5-50 µg/ml)

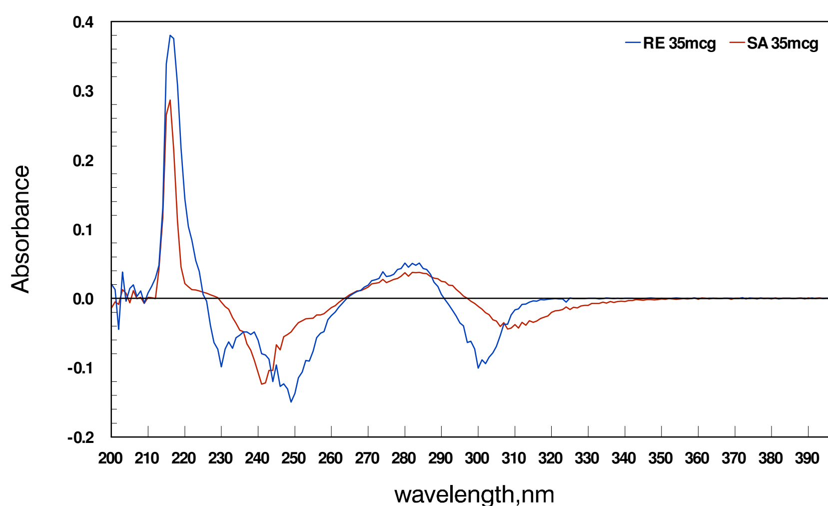


Fig.32. First derivative spectra for salicylic acid (35 µg/ml) resorcinol (35 µg/ml), indicating the zero - crossing points of each of them

By taking the zero-crossing points of resorcinol for the estimation of salicylic acid the following analytical parameters and predicted values were obtained (Table 4 and Table 5). The same procedure was followed for determination of resorcinol in the presence of salicylic acid and the results of it are presented in Table 4 and Table 6.

Table 4. Spectral and analytical parameters for both salicylic acid and resorcinol as calculated from the first derivative zero crossing points against the given concentrations of standard compounds at the selected wavelengths:

Compound	λ * (nm)	LC R ** (μg/ml)	Slope (b) ±SE***	Intercept (a) ±SE	r *	r^2 **	LOD † (μg/ml)	LOQ †† (μg/ml)
Salicylic acid	290.5 nm	5-50	-0.00019±0.00031	0.00068±0.00001	0.9991	0.998	1.4	4.6
	317.5 nm	5-50	0.00124±0.00069	-0.00079±0.00002	0.9968	0.994	2.6	8.7
Resorcinol	297.5 nm	5-50	0.00047±0.00106	-0.00180±0.00003	0.9986	0.997	1.7	5.8

* λ=wavelength of determination, ** LCR= linear calibration range, *** SE=standard error , * r= correlation coefficient
** r^2= determination coefficient, † LOD= limit of detection, †† LOQ= limit of quantification

Table 5. Actual and predicted amounts of salicylic acid given by applying first derivative zero crossing points spectrophotometric techniques for pure, synthetic mixtures with resorcinol and luna soap and dorin emulsion determined at 290.5 nm and 317.5 nm .

Component analyzed	Concentrations used (µg/ml)	Recovery at 290.5 nm			Recovery at 317.5 nm		
		µg/ml	%	*RSD	µg/ml	%	*RSD
Pure salicylic acid	5	5.07	101.4	3.8	6.27	125.3	16
	10	10.32	103.3	3.6	10.36	103.6	3.5
	15	15.13	100.9	1.9	15.04	100.3	1.0
	20	20.45	102.3	3.4	19.60	98.0	4.3
	25	23.51	94.0	1.3	22.63	90.5	3.7
	30	30.01	100.0	1.3	28.45	94.8	5.2
	35	34.74	99.3	0.6	35.78	102.2	1.7
	40	40.89	102.2	1.1	41.32	103.3	2.0
	45	44.62	99.2	0.8	45.85	101.9	0.6
	50	50.26	100.5	1.4	49.69	99.4	0.4
Salicylic acid in binary mixture with resorcinol	10(5)*	12.21	118.3	0.1	11.51	115.1	6.9
	10(10)	13.16	128.1	0.5	14.10	141.0	6.3
	5(10)	8.64	162.9	2.1	8.57	171.3	5.3
Dorin emulsion	10(5)	7.41	130.2	12.8	13.30	266.1	5.1
Luna soap	10(10)	12.27	109.7	0.3	15.26	152.6	3.4

* ( ) the concentrations of resorcinol in the studied mixtures in µg/ml

* RSD is the relative standard deviation

Table 6. Actual and predicted amounts of resorcinol given by applying first derivative zero crossing points spectrophotometric techniques for pure salicylic acid, synthetic mixtures with and luna soap and dorin emulsion determined at 297.5 nm

Component analyzed	Concentrations used (µg/ml)	Recovery at 297.5 nm		
		µg/ml	%	*RSD
Pure resorcinol	5	5.61	112.2	2.4
	10	10.23	102.3	3.9
	15	14.89	99.2	2.7
	20	19.47	97.4	3.5
	25	23.84	95.4	0.1
	30	29.77	99.2	2.6
	35	34.91	99.8	3.5
	40	41.44	103.6	2.5
	45	45.80	101.8	2.9
	50	49.05	98.1	3.5
Resorcinol in binary mixture with salicylic acid	5(10) *	6.00	120.1	7.6
	10(10)	11.36	113.5	5.3
	10(5)	11.74	117.8	6.2
Dorin emulsion	5(10)	4.56	91.1	4.3
Luna Soap	10(10)	6.44	64..4	5.4

* ( ) the concentrations of salicylic acid in the studied mixtures in µg/ml * RSD is the relative standard deviation

As a preliminary conclusion;

- the [1]D method is a sufficiently good method for analysis of individual compounds (of benzoic acid and salicylic acid) and their binary mixtures with a considerably good precision when applied in the same ranges of calibration domains. The variations and relative deviations from the mean observed is insignificant. Thus, [1]D method is a suitable method for simultaneous determination of benzoic acid and salicylic acid mixture.

-the [1]D method is a sufficiently good method for analysis of individual compounds but when applied in the same ranges of calibration domains of the studied drugs a pronounced variations and marked measurement errors appeared, as well as high relative deviations from the mean values for salicylic and resorcinol mixture. Thus [1]D method is not a suitable method for simultaneous determination of such binary mixture due to the high overlapping percentage (96.2 %) and the great background interferences in the pharmaceutical formulations.

4.3. Derivative ratio method

Calculations in this technique rely on the following basis;

Consider a mixture M of two components X and Y. If Beer's law is obeyed for both components over the whole wavelength range used and the path length is 1 cm, the absorption spectrum, of the mixture $A_{x,y}$ is defined by the Equation

$$A_{x,y} = \alpha_{xi} C_X + \beta_{Yi} C_Y \text{ ....[14]}$$

Where $A_{x,y}$ is the absorbance of the mixture at wavelength i; α_{Xi} and β_{Yi} are the A (1%, 1cm) of X and Y at a given wavelength i ; C_X and C_Y are the concentrations of X and Y respectively. If such equation 14 is divided by the corresponding equation for the spectrum of a standard solution of X (the same is correct for the component Y) of concentration C^o_X, the following Equation can be written.

$$A_{x,y} / \alpha_{Xi} C^o_X = C_X / C^o_X + C_Y \beta_{Yi} / C^o_X \alpha_{Xi} \text{ ....[15]}$$

Which can be simplified to

$$A_{x,y} / \alpha_{Xi} = C_X + C_Y (\beta_{Yi} / \alpha_{Xi}) \text{..[16]}$$

By plotting $A_{x,y} / \alpha_{Xi}$ as a function of β_{Yi} / α_{Xi} a straight line is obtained. The intercept of the straight line provides the value of C_X and the slope of the straight line is C_Y . To obtain the ratio β_{Yi} / αXi at each wavelength, the absorption spectra of equimolar standard solutions of Y and X are measured and the absorbance ratio at each wavelength is calculated. At the same time the differentiation of equation 15 to its first derivative, give the following equation:-

$$d/d\lambda \, (A_{Mi} / \alpha_{Xi} C^o_X) = (C_Y / C^o_X) \, d/d\lambda \, (\beta_{Yi} / \alpha_{Xi}) \text{..................[17]}$$

Equation 17 indicates that the derivative ratio spectrum of the mixture is dependent only on the values of C_Y and C^o_X and independent on the values of C_X in the mixture. A calibration graph is obtained by recording and storing the spectra of solution of pure Y at different concentrations, and the spectrum of a solution of pure X, of concentration C^o_X. The amplitudes of Y are then divided (wavelength by wavelength) by the corresponding amplitudes for X.

The ratio spectra thus obtained are then differentiated with respect to wavelength and the derivative values for given wavelength are plotted against C_Y to give a calibration graph. Applications of the method to the sample containing both X and Y, and use of the calibration graph, will then give the value of C_Y in the mixture. X can be determined by an analogous procedure. If the concentration of the standard (the divisor) is increased or decreased, the resulting first derivative values are proportionally decreased or increased, respectively, although the maxima and minima remain at the same wavelength. In consequence the ratio of the slopes of two calibration graphs must be equal to the inverse of the concentration ratio of the divisors

4.3.1. Benzoic acid & salicylic acid mixture

Benzoic acid

The first derivative ratio spectrum of benzoic acid was obtained by dividing the amplitude of the mixture spectrum by the amplitude of the spectrum of salicylic acid. For benzoic acid different divisors of salicylic acid concentrations were taken at various wavelengths however the most convenient divisor was 25 µg/ml (with 3 nm difference intervals) and 269.5 nm amplitude. By this the absorbitivity and concentration effects of salicylic acid was deleted (protected) from benzoic acid and the calculations were performed accordingly.

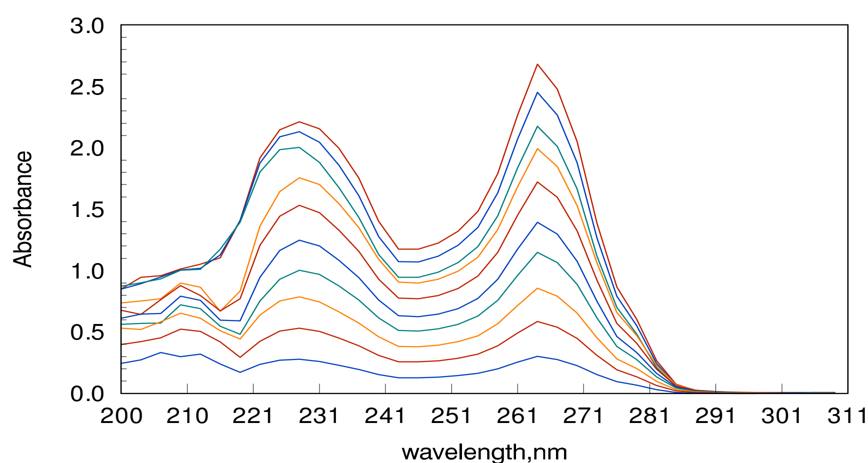


Fig.33. Benzoic acid spectral ratio (divisor is 25 µg/ml of salicylic acid at 3 nm difference intervals)

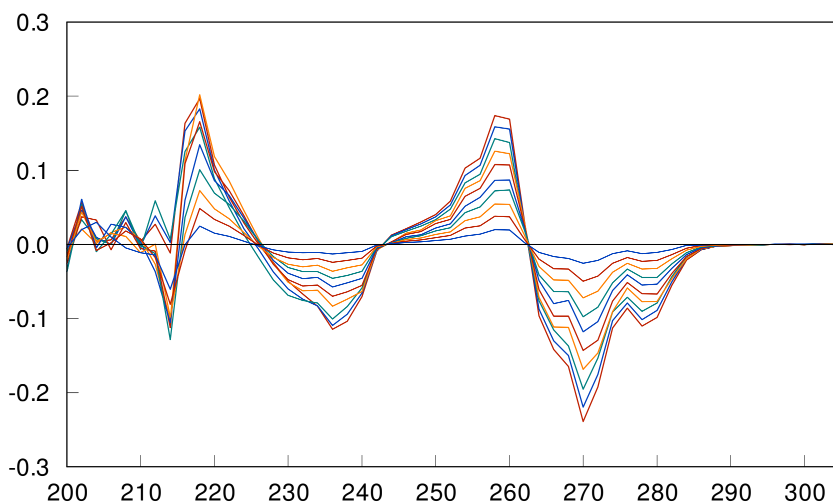


Fig.34. Benzoic acid derivative ratio (divisor is 25 µg/ml salicylic acid at 2 nm difference intervals)

Salicylic acid

Similar procedures were followed for salicylic acid as in case of benzoic acid. In the cases of salicylic acid the best divisor for calculation (values) was 50 µg/ml (with 4 nm differences) and amplitudes 292.5 nm and 308.5 nm. After having a critical look at different wave lengths the researcher resulted in the best and suitable values. These subsequently show that wave lengths and divisors have got their effect on the derivative ratio spectrum values of salicylic acid.

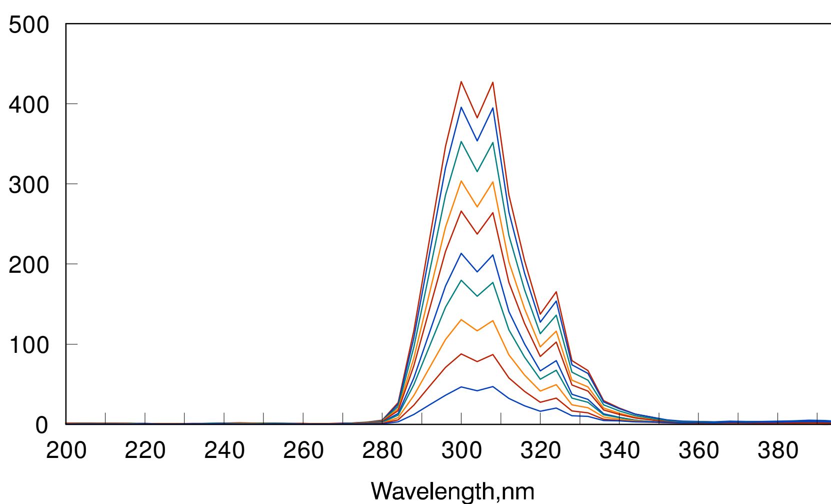


Fig.35. Salicylic acid spectral ratio (divisor is 50 µg/ml benzoic acid at 4 nm difference intervals)

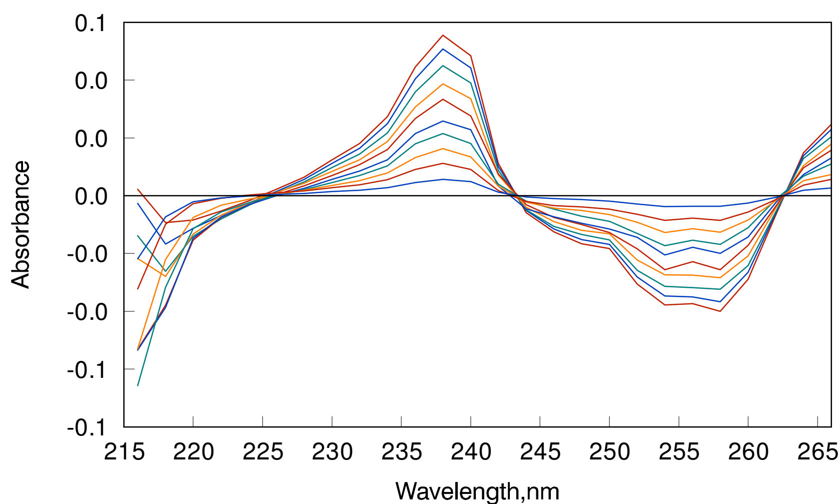


Fig.36. Salicylic acid derivative ratio (divisor is 50 µg/ml benzoic acid at 4 nm difference intervals).

Linear relationships between the derivative ratio amplitudes as each of benzoic and salicylic acid concentrations were obtained over the used concentration ranges (5-50 µg/ml).

Derivative ratio spectra values for benzoic acid at 269.5 nm and for salicylic acid at 292.5 nm and 308.5 nm were used for all calculations of concentrations of benzoic acid and salicylic acid in all the subsequent work: linear regression ranges, slopes, intercepts, correlation and determination coefficients, limits of detection (LOD, µg/ml) and limits of quantification. (LOQ, µg/ml) for both compounds are shown in the following tables (Tables 7-9).

Table 7. Spectral and analytical parameters for both benzoic acid and salicylic acid as calculated from the derivative ratio amplitudes against the given concentrations of standard compounds at the selected wavelengths:

Compound	λ * (nm)	LC R ** (µg/ml)	Slope (b) ±SE***	Intercept (a) ±SE	r *	r^2 **	LOD ‡ (µg/ml)	LOQ‡‡ (µg/ml)
Benzoic acid	269.5 nm	5-50	-0.00070±0.00150	-0.00450±0.00005	0.9995	0.999	1.0	3.4
Salcylic acid	292.5 nm	5-50	0.13754±0.19777	0.58725±0.00638	0.9995	0.999	1.0	3.4
	308.5 nm	5-50	-0.25672±0.23140	-0.70914±0.00746	0.9996	0.999	0.9	3.3

* λ=wavelength of determination , ** LCR= linear calibration range , *** SE=standard error , * r= correlation coefficient

** r^2= determination coefficient, ‡ LOD= limit of detection, ‡‡ LOQ= limit of quantification

Table.8. Actual and predicted amounts of benzoic acid of given by applying derivative ratio spectrophotometric techniques for pure, synthetic mixtures with salicylic acid and commercial white field ointment (peak heights measured at 269.5 nm).

Component analyzed	Concentrations used (µg/ml)	Recovery		
		µg/ml	%	*RSD
Pure benzoic acid	5	5.19	103.9	2.6
	10	10.19	101.9	1.3
	15	14.73	98.2	1.3
	20	20.20	101.0	1.8
	25	24.91	99.6	0.3
	30	29.69	99.0	0.7
	35	34.88	99.7	0.3
	40	40.17	100.4	0.3
	45	45.17	100.4	0.6
	50	49.58	99.2	1.3
Benzoic acid in binary mixture with salicylic acid	50(10) *	50.22	100.4	0.3
	45(15)	45.10	100.2	0.2
	40(20)	39.94	99.9	2.1
	35(25)	34.47	98.5	1.1
	30(30)	29.38	97.9	1.5
	25(35)	24.55	98.2	1.3
	20(40)	20.86	104.3	1.9
	15(45)	15.53	103.5	0.4
	10(50)	10.25	102.5	4.9
White field	40(20)	40.17	100.4	3.1

* ( ) the concentrations of salicylic acid in the studied mixtures in µg/ml

* RSD is the relative standard deviation

Table 9. Actual and predicted amounts of salicylic acid given by applying first derivative ratio spectrophotometric techniques for pure, synthetic mixtures with benzoic acid and white field (peak height measured at 292.5 nm and 308.5 nm)

Component analyzed	Concentrations used(µg/ml)	Recovery F at 292.5 nm			Recovery at 308.5 nm		
		µg/ml	%	*RSD	µg/ml	%	*RSD
pure salicylic acid	5	4.90	98.2	1.3	4.98	99.6	0.4
	10	9.93	99.0	0.3	10.05	100.1	1.1
	15	14.81	98.7	1.3	14.74	100.3	1.5
	20	20.50	102.2	2.1	19.97	99.9	2.5
	25	24.26	97.7	0.6	24.49	98.0	1.8
	30	30.51	101.7	1.2	29.86	99.5	1.1
	35	34.99	99.8	0.3	35.03	100.1	0.2
	40	40.63	101.5	0.8	40.15	100.4	0.6
	45	45.28	100.7	0.4	45.41	100.9	1.4
	50	49.20	98.4	0.8	49.08	99.1	1.3
salicylic acid in binary mixture with benzoic acid	10(50) *	9.94	99.5	3.5	10.05	100.5	1.5
	15(45)	14.73	98.0	0.9	15.01	100.1	1.1
	20(40)	20.12	100.3	2.7	20.30	101.5	0.5
	25(35)	24.83	98.7	2.3	24.67	98.7	1.9
	30(30)	29.77	99.2	0.6	29.93	99.8	0.4
	35(25)	34.78	99.0	3.0	35.25	100.7	0.9
	40(20)	39.92	99.8	2.0	40.66	101.6	0.7
	45(15)	45.03	99.8	1.8	44.91	99.8	1.9
	50(10)	48.85	98.9	1.1	46.28	99.5	0.9
White field	20(40)	19.20	96.4	0.3	19.81	99.1	2.0

* ( ) the concentrations of benzoic acid in the studied mixtures in µg/ml

*RSD is the relative standard deviation

4.3.2. Salicylic acid & resorcinol mixture

Salicylic acid

As mentioned before the first derivative ratio spectrum of salicylic acid was obtained by dividing the amplitude of the mixture spectrum by the amplitude of the spectrum of resorcinol. Different divisors of resorcinol concentration were taken at various wavelengths. However the most convenient divisor was 25 µg/ml (with 3 nm difference intervals) and 314.5 nm and 323.5 nm wavelengths were selected for determinations. By this the

absorbitivity and concentration effects of resorcinol was deleted (protected) from salicylic acid and the calculations were performed accordingly.

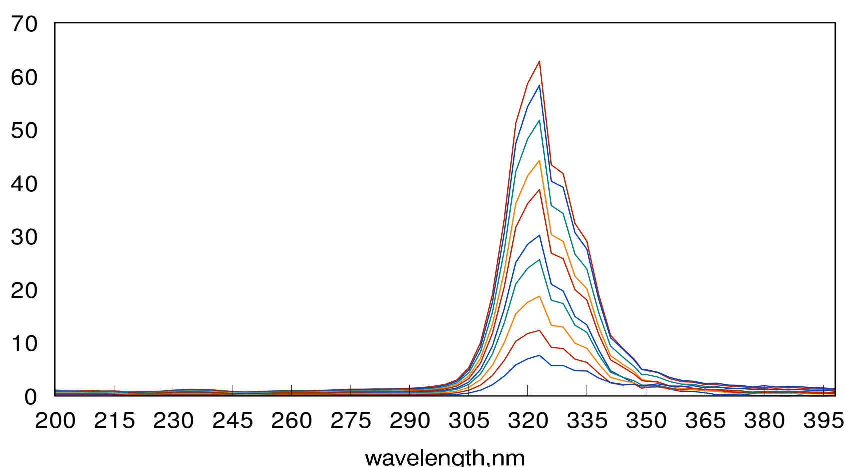


Fig.37. Salicylic acid spectral ratio (divisor is 25 µg/ml resorcinol at 3 nm difference intervals)

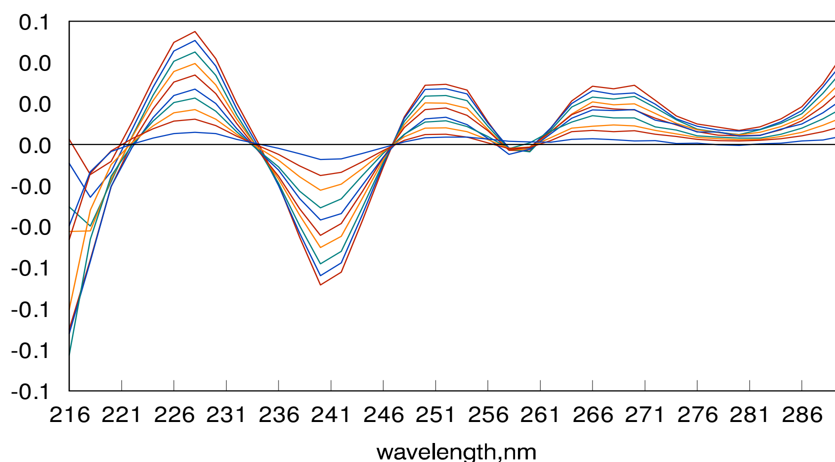


Fig.38. Salicylic acid derivative ratio (divisor is 25 µg/ml resorcinol at 3 nm difference intervals)

Resorcinol

Similar procedures were followed for resorcinol as that of salicylic acid. In the cases of resorcinol the best divisor for calculation (values) was 5 µg/ml (with 1 nm differences) and amplitudes 242.5 nm and 302.5 nm. After having a critical look at different wave lengths the researcher resulted in the best and suitable values. These subsequently show that wavelengths and divisors have got their effect on derivative ratio spectrum values of salicylic acid.

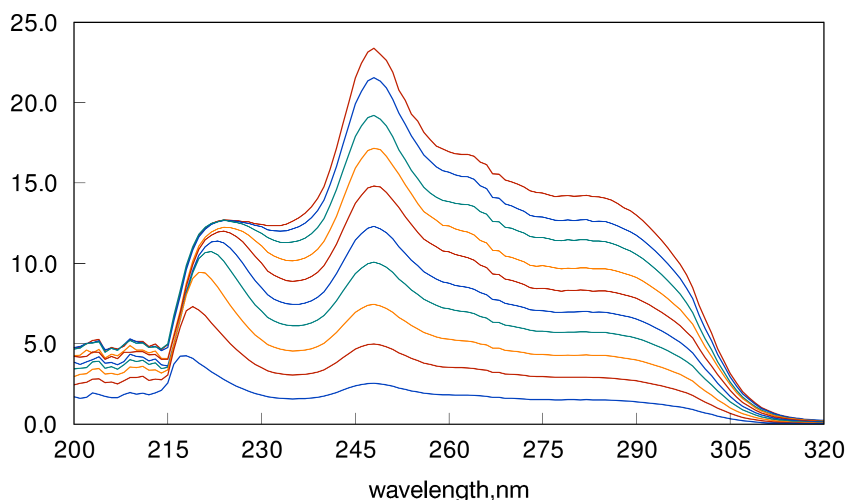


Fig.39.Resorcinol spectral ratio (divisor is 5 µg/ml salicylic acid at 1 nm difference intervals)

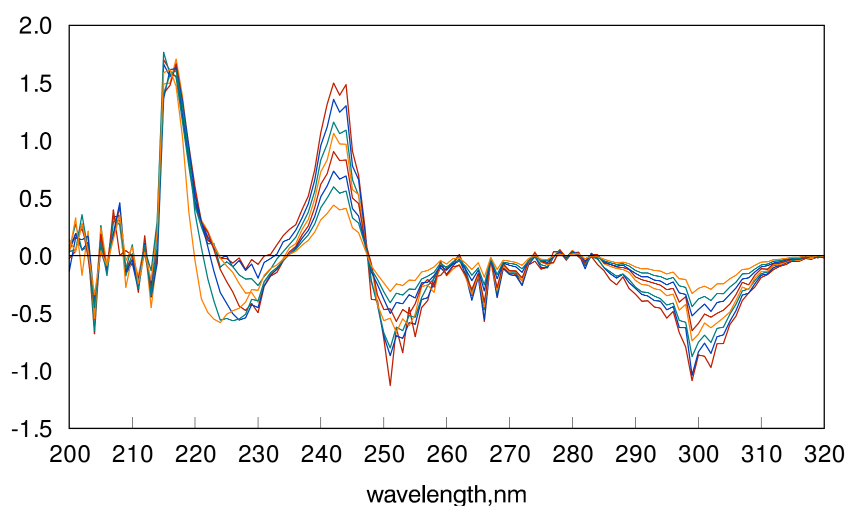


Fig.40. Resorcinol derivative ratio (divisor is 5 µg/ml salicylic acid at 1nm difference interval)

Derivative ratio spectra values for salicylic acid at 314.5 nm, 323.5 nm, and that for resorcinol at, 302.5 nm were considered and concentrations of both compounds were calculated by their respective regressive equations using derivative ratio spectra values just obtained. Comparison of the known spiked amount and the calculated counter parts of the two compounds yielded the two compounds recoveries shown in table 10-12. The LOD, LOQ, and RSD of each compounds and samples were calculated and shown in the table 10.

Table 10. Spectral and analytical parameters for both salicylic acid and resorcinol as calculated from the derivative ratio amplitudes against the given concentrations of standard compounds at the selected wavelengths:

Compound	λ * (nm)	LC R ** (μg/ml)	Slope (b) ±SE***	Intercept (a) ±SE	r *	r^2 **	LOD ☨ (μg/ml)	LOQ☨☨ (μg/ml)
Salicylic acid	242.5 nm	5-50	0.01026±0.05667	0.12149±0.00183	0.9991	0.998	1.4	4.7
	302.5 nm	5-50	0.17509±0.07501	-0.13587±0.00242	0.9988	0.998	1.7	5.5
Resorcinol	242.5 nm	5-50	-0.00707±0.01100	0.03001±0.00036	0.9994	0.999	1.1	3.7
	302.5 nm	5-50	-0.00453±0.01421	-0.01876±0.00046	0.9976	0.995	2.3	7.6

* λ=wavelength of determination , ** LCR= linear calibration range , *** SE=standard error , * r= correlation coefficient
** r^2= determination coefficient, ☨ LOD= limit of detection, ☨☨ LOQ= limit of quantification

Table 11. Actual and predicted amounts of salicylic acid given by applying derivative ratio spectrophotometric techniques for pure, synthetic mixtures with resorcinol and, Dorin emulsion, and luna soap (peak height measured at 314.5 nm and 323.5 nm)

Component analyzed	Concentrations used (µg/ml)	Recovery at 314.5 nm			Recovery at 323.5 nm		
		µg/ml	%	∗RSD	µg/ml	%	∗RSD
Pure salicylic acid	5	5.24	104.9	4.5	4.92	98.4	0.1
	10	9.96	99.7	2.1	9.64	96.4	1.8
	15	14.87	99.0	1.7	14.66	97.7	0.5
	20	20.00	100.0	0.2	20.06	100.3	0.6
	25	24.49	98.0	1.9	25.65	102.6	1.4
	30	30.06	100.2	1.3	30.39	101.3	1.7
	35	34.71	99.2	1.1	35.49	101.4	0.7
	40	40.54	101.4	0.8	40.60	101.5	0.8
	45	45.79	101.7	0.5	45.50	101.1	1.2
	50	49.38	98.8	0.9	49.05	98.1	1.3
salicylic acid in binary mixture with resorcinol	10(5)*	9.86	98.6	0.2	9.88	98.8	0.8
	10(10)	10.39	103.9	1.9	10.12	101.2	0.9
	5(10)	4.96	99.1	1.3	5.07	101.3	1.2
Dorin emulsion	10(5)	10.12	101.2	1.1	10.14	101.4	1.8
Luna Soap	10(10)	10.09	100.9	1.7	10.15	101.5	1.6

* ( ) the concentrations of resorcinol in the studied mixtures in µg/ml

∗ RSD is the relative standard deviation

Table 12. Actual and predicted amounts of resorcinol given by applying derivative ratio spectrophotometric techniques for pure, synthetic mixtures with salicylic acid and , Dorin,and soap (Peak height measured at 242.5 nm and 302.5 nm)

Component analyzed	Concentrations used(µg/ml)	Recovery at 242.5 nm			Recovery at 302.5 nm		
		µg/ml	(%)	∗ RSD	µg/ml	(%)	∗ RSD
Pure resorcinol	5	5.12	102.5	1.7	5.15	103.0	2.7
	10	9.95	99.5	1.8	10.13	101.3	3.1
	15	14.83	98.9	0.8	15.00	100.0	0.6
	20	20.15	100.7	1.9	20.02	100.1	0.8
	25	24.66	98.6	2.0	25.18	100.7	1.9
	30	30.35	101.2	0.8	29.47	98.2	1.8
	35	35.02	100.1	1.3	35.03	100.1	1.6
	40	38.81	97.0	2.2	40.03	100.1	0.5
	45	45.40	100.9	1.2	44.97	99.9	0.8
	50	50.11	100.2	1.9	50.82	101.6	2.1
Resorcinol in binary mixture with salicylic acid	5(10) *	5.07	101.4	1.7	4.92	98.5	1.6
	10(10)	10.19	101.9	1.4	10.20	102.0	1.4
	10(5)	9.94	99.4	1.5	9.62	96.2	0.7
Dorin emulsion	5(10)	5.01	100.2	0.6	4.91	98.2	1.4
Luna soap	10(10)	9.81	98.1	2.1	10.11	101.1	1.1

* ( ) the concentrations of salicylic acid in the studied mixtures in µg/ml

∗ RSD is the relative standard deviation

As a preliminary conclusion,

- the ^{1}D ratio method is a sufficiently good method for analysis of individual compounds (of benzoic acid and salicylic acid, and salicylic acid and resorcinol) and their binary mixtures with a considerably good precision when applied in the same ranges of calibration domains. The variations and relative deviations from the mean observed is insignificant. Thus, ^{1}D ratio method is a suitable method for simultaneous determination of both benzoic acid and salicylic acid mixture, and salicylic acid and resorcinol mixtures.

4.4. Classical Least square method (CLS)

4.4.1. Calibration step

The purpose of the calibration step is to make a standard relationship between the response (absorbance) and concentration. The absorbencies of well known concentrations is measured at wide range of wavelengths (200-400 nm).

It is clear that the relationship to be set will strongly help to predict the concentration of unknown analyte to be analyzed. In a univariate case we may take one Y (response component) for each X value (concentration ingredient). Unlike to this in CLS case many Y components that measured under the same conditions for many X values are treated.

In this process, to attain the suitable calibration for the entire data (matrix) many steps are followed. Finally the efficiency of the calibration step is tested and the precision of determinations will be seen.

The calibration is done for pure compounds benzoic acid, salicylic acid and resorcinol. In all the cases about 10 rows and about 200 columns matrices are used. The calibration step done by using the zero order absorbencies. Thus the steps to be followed are the same in all compounds studied.

When calibration set of (m) samples of known content (pure compound) with (n) spectral variables are measured, the relationship is given by the equation :

$$A_n^m = C_L^m . K_n^L + E_n^m$$ **...................... [18]**

A_n^m = the m x n matrix of calibration spectra

C_L^m = m x L matrix of component concentrations

K_n^L = the L x n matrix of regression coefficients (slope).

E_n^m = the m x n matrix of spectral errors (residuals not fitted by the model)

For ideal cases (no residuals are given), and the equation becomes

$A = KC$ or $K = AC^{-1}$.......................... [19]

Dividing by C^{t}, where C^{t} is the transposed C matrix, the equation becomes

$$\frac{K}{C^{t}} = \frac{AC^{-1}}{C^{t}}$$ .... **[20]** Or after rearrangement

$K= (A. C^{t}) (C. C^{t})^{-1}$………………… [21]

Accordingly and by the same manner, dividing by K^{t} the equation becomes

$C_{st} = (A_{st}.K^{t}) (K. K^{t})^{-1}$…………………… [22]

Equations (21 and 22) are the main equations used for calculations in the calibration step.

4.4.2. Testing step

In this part of estimation, the spectra of pure compounds used in the calibration stage are replaced by the responses given by laboratory prepared binary mixtures (of well known composition) of the same pure compounds, to test the efficiency of the given model for the prediction of the individual concentrations in the tested binary mixtures of the studied compounds. Analysis is now based on the spectra given by the tested binary mixtures by using the same equations stated in the calibration stage as:

$C_{s} = A_{s}K^{t}_{s} (K_{s}K^{t}_{s})-^{1}$................................ [23]

Where C_{s}=concentration matrix of the given binary ($C_{1,2}$)

A_{s} = Responses (Absorption) matrix of the same mixture and

K_{s} = slopes matrix of the given mixture ($K_{1, 2}$ in the case of binary mixtures)

4.4.3. Prediction step

In this step the concentrations of dosage forms of mixtures are analyzed. Analysis is based on the spectrum (A_{un}) of the unknown samples by using the following equation:-

$$\mathbf{C_{un} = (A_{un}K^t_s)\ (K_s.K^t_s)^{-1}} \quad \textbf{[24]}$$

where C_{un} is the vector of sought for concentrations.

According to the previous steps, several laboratory prepared mixtures were subjected to the analysis by the proposed technique in order to confirm the suitability of the calibration models for determination of the studied compounds in the pharmaceutical proportions.

Tables 13 and 14 summarize the results obtained for the pure compounds suggested synthetic binary mixtures and the pharmaceutical proportions.

As can be seen, the concentrations predicted by the models are considerably close to the real concentrations. The recoveries in most cases were satisfactory and the relative deviations between the estimated and true concentrations were found within the normal levels.

Table 13. Actual and predicted amounts of salicylic acid given by applying CLS spectrophotometric techniques for pure, synthetic mixtures with benzoic acid and whitefield ointment (232-400 nm).

Component analyzed	BA* Conc. used (µg/ml)	Recovery			SA** Conc.used (µg/ml)	Recovery		
		µg/ml	(%)	∗ RSD		µg/ml	(%)	∗ RSD
Pure component	10	10.26	102.6	4.3	10	10.12	101.2	0.9
	15	15.06	100.4	5.2	15	15.10	100.7	1.0
	20	20.63	103.2	3.6	20	20.00	100.0	3.9
	25	25.36	101.5	3.1	25	24.53	98.1	2.1
	30	31.07	103.6	1.4	30	30.71	102.4	1.3
	35	35.17	100.5	3.1	35	35.02	100.1	0.5
	40	39.39	98.5	2.9	40	40.59	101.5	1.2
	45	44.75	99.4	0.9	45	45.23	100.5	1.0
	50	48.14	96.3	0.2	50	48.73	97.5	1.2
Synthetic mixtures	50(10)	44.18	88.4	1.2	10(50)	9.98	99.8	3.9
	45(15)	40.15	89.2	1.9	15(45)	15.09	100.6	3.2
	40(20)	35.61	89.0	2.1	20(40)	19.83	99.1	3.4
	35(25)	30.87	88.2	4.8	25(35)	25.07	100.3	2.8
	30(30)	25.15	83.8	3.1	30(30)	30.21	100.7	1.1
	25(35)	21.11	84.4	2.2	35(25)	34.86	99.6	2.3
	20(40)	15.15	75.7	4.5	40(20)	39.70	99.3	3.7
	15(45)	10.65	71.0	6.2	45(15)	45.24	100.5	1.7
	10(50)	7.06	70.6	8.3	50(10)	49.40	98.8	4.7
White field	40(20)	42.95	107.4	10	20(40)	20.10	100.5	2.8

*benzoic acid, ** salicylic acid

∗ RSD is the relative standard deviation

Table 14. Actual and predicted amounts of salicylic acid (SA) and resorcinol (RE) given by applying CLS spectrophotometric techniques for pure, synthetic mixtures, Dorin emulsion, and Luna soap. (250 nm-400 nm)

Component analyzed	SA Conc. used (µg/ml)	Found			RE conc. Conc.used µg/ml	Recovery		
		µg/ml	(%)	* RSD		µg/ml	(%)	* RSD
Pure component	5	5.25	106.8	5.0	5	5.28	105.7	2.0
	10	9.78	100.2	5.1	10	10.28	102.8	1.9
	15	14.85	99.8	0.9	15	15.27	101.8	0.2
	20	20.41	103.1	3.9	20	20.54	102.7	0.4
	25	23.67	97.1	2.2	25	25.11	100.4	0.3
	30	30.71	101.8	1.0	30	30.15	100.5	1.6
	35	35.03	99.5	0.4	35	35.06	100.2	0.8
	40	40.66	101.2	1.1	40	40.15	100.4	1.5
	45	45.49	100.9	0.9	45	44.90	99.7	0.8
	50	49.15	98.2	0.7	50	49.42	98.8	0.1
Synthetic mixtures	10(5)	10.23	99.7	3.3	5(10)	4.98	99.5	3.2
	10(10)	10.45	101.5	1.0	10(10)	10.14	101.4	2.1
	5(10)	5.17	100.8	0.6	10(5)	10.19	101.9	1.6
Dorin emulsion	10(5)	10.25	102.5	2.0	5(10)	4.98	99.6	0.6
Luna soap	10(10)	10.45	104.5	1.8	10(10)	10.14	101.4	0.4

* RSD is the relative standard deviation

As a preliminary conclusion;

-although the CLS method is a sufficiently good method for analysis of individual compounds, the method is failed to determine the proper concentrations of components of benzoic acid in the laboratory preparations and commercial formulations analyzed. Thus the method is failed to determine the binary combination of benzoic acid and salicylic acid.

- the CLS method is a sufficiently good method for analysis of individual compounds (salicylic acid and resorcinol) and their binary mixtures with a considerably good precision when applied in the same ranges of calibration domains. The variations and relative deviations from the mean observed is insignificant. Thus, CLS method is a suitable method for simultaneous determination of salicylic acid and resorcinol mixture.

4.5. Principal component regression method

PCR predicts response variables from factors underlying the predictor variables. In stead of directly using absorbance values, we use in PCR data matrix containing the coordinates of each spectrum each of the axes of the new coordinate system. The new coordinates are the projections of the spectra on to the basis vector. These projections are easily computed from the following equation.

$$\mathbf{A_{proj} = V^t_c.A} \text{.............................. [25]}$$

Where A_{proj} = matrix containing new coordinates.

A = original absorbance matrix as

V_c=matrix containing the basis vectors (one column for each factor retained).

Accordingly the main equation including new responses and concentration components can be written as

$$\mathbf{C= F.A_{proj}} \text{.................................... [26]}$$

Where C is the concentration components and F is the regression coefficient matrix of the new coordinates .Thus

$$\mathbf{F \text{ (similar to K in CLS)} = (C.A^t{}_{.proj})(A_{proj}.A^t{}_{Proj})^{-1}} \text{.................[27]}$$

Then the calculated F values are used to predict the concentrations in an unknown sample from its measured spectrum as:

$$\mathbf{C_{un} = F.V^t_c.A_{un}} \text{.......................... [28]}$$

or

$$\mathbf{C_{un} = F_{cal.}.A_{un}} \text{........................... [29]}$$

Note that, F_{cal} has exactly the same properties and format as K_{cal} in CLS. It has one column for each wavelength in the spectrum as one raw for each component in the mixture.

By the same manner stated in CLS method, the same laboratory prepared mixtures were subjected to the analysis by the proposed PCR technique in order to confirm the efficiency of the given model for the subsequent determination of the studied compounds in the pharmaceutical preparations.

Tables 15- 17 summarize the results obtained for the pure compounds, laboratory prepared mixtures and pharmaceutical preparations.

Table 15. Predicted concentrations of pure benzoic acid, salicylic acid and resorcinol by using PCR technique

Concentrations (µg/ml)	Benzoic acid		Salicylic acid		Resorcinol	
	Recovery (µg/ml)	%±RE*	Recovery (µg/ml)	%±RE	Recovery (µg/ml)	%±RE
5	4.92	98.4±1.96	4.94	98.8±1.11	4.93	98.6±1.45
10	9.87	98.7±1.88	9.97	99.7±1.23	9.94	99.4±1.98
15	14.80	98.7±1.86	14.95	99.7±1.96	14.88	99.2±0.76
20	19.61	98.1±1.23	20.28	101.4±+1.77	20.07	100.3±0.49
25	24.68	98.7±1.67	25.17	100.7±1.98	24.89	99.6±1.12
30	29.70	99.0±1.33	29.76	99.2±1.45	29.90	99.7±1.56
35	35.20	100.6±1.74	34.89	99.7±2.00	34.70	99.1±1.77
40	40.09	100.2±1.05	40.36	100.9±+1.56	40.30	100.8±0.98
45	44.86	99.7±0.98	45.59	101.3±1.14	45.80	101.8±0.88
50	50.46	100.9±0.92	49.41	98.8±1.78	49.56	99.1±1.44

*Relative error as calculated from the corresponding calibration model (n=3)

Table 16. Predicted concentrations of benzoic acid and salicylic in laboratory prepared mixtures and in white field ointment by using PCR technique:

Benzoic acid			Salicylic acid		
Concentrations	Recovery	%±RE*	Concentrations	Recovery	%±RE
50	49.22	98.4±1.55	10	10.12	101.2±1.60
45	44.50	98.9±1.76	15	15.08	100.6±1.67
40	39.64	99.1±1.92	20	20.15	100.8±1.81
35	35.12	100.3±1.95	25	24.96	99.8±1.01
30	30.14	100.5±1.04	30	30.01	100.0±1.22
25	24.89	99.6±1.44	35	34.38	98.2±0.89
20	19.89	99.5±0.96	40	39.34	98.4±1.56
15	15.10	100.7±0.93	45	44.40	98.7±0.92
10	9.97	99.7±1.33	50	49.67	99.3±1.22
White field ointment:					
40	40.37	100.9±1.83	20	19.64	98.2±1.23

*Relative error as calculated from the corresponding calibration model (n=3)

Table 17. Predicted concentrations of salicylic acid and resorcinol in laboratory prepared mixtures and in luna soap and dorin emulsion by using PCR technique;

Salicylic acid			Resorcinol		
Concentrations	Recovery	%±RE*	Concentrations	Recovery	%±RE
10	10.20	102.0±2.05	5	4.92	98.3±1.93
10	10.18	101.8±1.57	10	9.86	98.6±2.11
5	4.98	99.7±1.44	10	9.92	99.2±1.78
Dorin emulsion:					
10	9.93	99.3±1.84	5	5.06	101.1±1.15
Luna soap:					
10	10.14	101.4±1.78	10	10.17	101.7±1.76

*Relative error as calculated from the corresponding calibration model (n=3)

The concentrations predicted by the PCR method are quite close to the real concentrations used and the recoveries in all cases are too satisfactory and the relative deviations between the estimated and true values were found in good agreement with each other

As a preliminary conclusion,

- the PCR method is a sufficiently good method for analysis of individual compounds (of benzoic acid and salicylic acid, and salicylic acid and resorcinol) and their binary mixtures with a considerably good precision when applied in the same ranges of calibration domains. The variations and relative deviations from the mean observed is insignificant. Thus, PCR method is a suitable method for simultaneous determination of both benzoic acid and salicylic acid mixture, and salicylic acid and resorcinol mixtures.

4.6. Comparision of the results from the proposal methods with each other and with the official procedures

Tables 18 and 19 below show the analyses of the results obtained for pure, laboratory prepared and commercial dosage forms of white field between ^{1}D, ^{1}D ratio,CLS and PCR methods the official method. The accuracy and precision were analyzed using t-test and F-test. Accordingly the result shows except CLS the rest method complies or agrees with the official method since there is no significant difference was observed. The calculated t-values and F-values are all less than the tabulated (critical) t –and F values respectively.

It can be observed from these sets of results that the compounds under investigation could be analyzed by the suggested methods ^{1}D, ^{1}D ratio and PCR with a good limit of precision. But the multivariate procedure PCR is more suitable for such type of analysis and gives more precise results.

Table 20 shows the intra comparison of the results obtained for laboratory prepared and commercial dosage forms of luna soap and dorin emulsion with in (between) ^{1}D, ^{1}D ratio, CLS and PCR methods using the recoveries and RSD values. Similar to white field, the factorized multivariate calibration models (PCR) allow a more significant reduction of errors in relation to the other used methods. Thus PCR has got more advantage from the suggested methods.

Table 18. Statistical analysis of the results obtained for pure benzoic acid, salicylic acid and resorcinol by the proposed methods using percentage found at 20 µg/ml.

Component analyzed	Statistical parameters	Reported(official) methods	^{1}D (zero-crossing)	^{1}D (derivative ratio)	CLS	PCR
Pure benzoic acid	X	99.9	99.4	101.0	103.2	98.1
	± S	1	2.1	1.8	3.6	1.2
	n	3	3	3	3	3
	S^2	1	4.41	3.24	12.96	1.44
	t	-	0.36	0.76	0.88	1.62
	F	-	4.41	3.24	12.96	1.44
Pure salicylic acid	X	99.7	100.4	99.9	100.0	101.4
	± S	1	3.1	2.5	3.9	1.8
	n	3	3	3	3	3
	S^2	1	9.61	6.25	15.21	3.24
	t	-	0.37	0.11	0.11	1.16
	F	-	9.61	6.25	15.21	3.24
Pure resorcinol	X	99.9	97.4	100.7	100.8	100.3
	± S	1	3.5	1.9	3.9	0.5
	n	3	3	3	3	3
	S^2	1	12.5	3.61	15.21	0.25
	t	-	0.97	0.53	0.32	0.51
	F	-	12.5	3.61	15.21	0.25

Theoretical values at 95 % confidence limit are t =2.776, and F =19 % (n_1 =3, n_2=3)
X = mean n = number of observations S = Relative Standard deviation S^2 = variance

Table19. Statistical analysis of the results obtained for laboratory prepared and commercial dosage forms of whitefield by the proposed methods using percentage found at 40 µg/ml for benzoic acid and 20 µg/ml for salicylic acid.

Component analyzed	SP*	Reported (official) methods	^{1}D(zero-crossing)		^{1}D (derivative ratio)		CLS		PCR	
			Lab prepaed	whitefield	Lab prepaed	whitefield	Lab prepaed	whitefield	Lab prepaed	whitefield
benzoic acid	X	101.8	100.9	100.2	99.1	100.4	89.0	107.4	99.1	100.9
	± S	1.7	2.9	1.3	2.1	3.1	2.1	10	1.9	1.8
	n	3	3	3	3	3	3	3	3	3
	S^2	2.89	8.41	1.69	4.41	9.61	4.41	100	3.61	3.24
	t	-	0.95	1.38	1.32	0.61	7.76	7.11	1.77	0.61
	F	-	2.91	1.00	2.52	3.32	4.41	34.6	1.25	1.12
salicylic acid	$\bar{X}$	99.8	101.4	99.3	101.5	99.1	99.1	100.5	100.8	98.2
	± S	1.8	0.9	0.5	0.5	2.0	3.4	2.8	1.8	1.2
	n	3	3	3	3	3	3	3	3	3
	S^2	3.24	0.81	0.25	0.25	4.00	11.56	7.84	1.56	1.44
	t	-	1.68	0.63	2.15	0.44	0.28	0.33	0.61	1.44
	F	-	4.00	12.96	1.12	1.24	3.57	2.42	1.00	2.25

*statistical parameters
Theoretical values at 95 % confidence limit are t =2.776, and F =19 % (n_1 =3 , n_2=3)
X = mean n = number of observations S = Relative Standard deviation S^2 = variance

Table 20. Statistical analyses of the results obtained for laboratory prepared and commercial dosage forms of luna soap and dorin emulsion (an intra-comparison) between the proposed CLS, PCR, and derivative methods found at dosage form concentrations.

Component analyzed	SP*	^{1}D(zero-crossing)				^{1}D (derivative ratio)				CLS				PCR			
		Lab prepaed		Dosage form		Lab prepaed		Dosage form		Lab prepaed		Dosage form		Lab prepaed		Dosage form	
		Luna soap	Dorin emulsion	Luna soap	Dorin emulsion	Luna soap	Dorin emulsion	Luna soap	Dorin emulsion	Luna soap	Dorin emulsion	Luna soap	Dorin emulsion	Luna soap	Dorin emulsion	Luna soap	Dorin emulsion
salicylic acid	X	128.1	118.3	109.7	130.2	103.9	98.6	100.9	101.2	101.5	99.7	104.5	102.5	101.8	102.0	101.4	99.3
	S	0.5	0.1	0.3	12.8	1.9	0.2	1.7	1.1	1.0	3.3	1.8	2.0	1.6	2.1	1.8	1.8
resocinol	X	113.5	120.1	64.4	91.1	101.9	99.4	98.1	100.2	101.4	99.5	101.4	99.6	98.6	98.3	101.7	101.1
	S	1.3	3.6	3.4	4.3	1.4	1.5	2.1	0.6	2.1	3.2	0.4	0.6	101.7	1.8	1.8	1.2

*statistical parameters

Theoretical values at 95 % confidence limit are X = mean, S = Relative standard deviation

5. GENERAL CONCLUSSION

The contents of several laboratory prepared mixtures and commercial dosage forms were simultaneously determined using chemometrics assisted UV-spectrophtometric measurements together with ^{1}D, ^{1}D ratio, CLS and PCR calibration analysis.

From the proposed methods good recoveries were obtained in ^{1}D, ^{1}D ratio and PCR for the benzoic and salicylic acid mixture (white field ointment). Three of them exhibited good accuracy, precision, and sensitivity to be applied for such binary combination. A comparison of these results with the previously obtained results for the same mixtures by using the proposed procedures revealed that there is no significant differences between them as shown by the compared t-and F-values for each pair of them to be applied efficiently for determination of studied drug simultaneously in the binary mixture as well as in the commercial dosage forms with satisfactory precision and accuracy. However CLS is failed to be applied because of poor recoveries and significant difference observed with the official method, may be due to the high background interferences.

In the cases of salicylic acid and resorcinol mixtures (luna soap and dorin emulsions) from the proposed methods good recoveries were obtained in ^{1}D ratio, CLS and PCR methods. The recoveries and RSD values observed reveal that PCR method is more accurate and precise over the rest methods. However the ^{1}D method is not good enough to be applied for such combinations due to great degree of overlapping.

The PCR technique has got superiority over the rest chemometrc techniques for all compounds studied. The proposed PCR method is a sufficiently precise and accurate as indicated by the validation results, and suitable for quality control laboratories, where the economy and time are important factors.

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